

INTESTINAL
MACROMOLECULAR TRANSMISSION
AND SOME
IMMUNOLOGICAL IMPLICATIONS

Esbjörn Telemo



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AND
SOME IMMUNOLOGICAL IMPLICATIONS

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"With one hand waving free"

Bob Dylan

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This thesis is based on the following publications which will be referred to by their roman numerals, I-V, and on some additional unpublished observations.

- I. Proteolytic Activity as a Regulator of the Transmission of Orally Fed Proteins from the Gut to the Blood Serum in the Suckling Rat.
E. Telemo, B.R. Weström, B.W. Karlsson
Biol. Neonate 41: 85-93 (1982).
- II. Intestinal transmission of BSA, FITC-Dextran and bovine IgG during postnatal development in the rat.
E. Telemo, B. Weström, G. Ekström, B.W. Karlsson
Submitted, Biol. Neonate 1986.
- III. The Passage of Orally Fed Proteins from Mother to Foetus in the Rat.
G.M.K. Dahl, E. Telemo, B.R. Weström, I. Jakobsson, T. Lindberg and B.W. Karlsson.
Comp. Biochem. Physiol. 77A 2:1984 199-201
- IV. Transfer of Orally or Intravenously Administered Proteins to the Milk of the Lactating Rat.
E. Telemo, B. Weström, G. Dahl and B. Karlsson
J. Ped. Gastroent. Nutr. 5: 305-309 1986
- V. Maternal dietary antigens and the immune response of the offspring in the Guinea pig.
E. Telemo, I. Jakobsson, B. Weström & Folkesson H.
Submitted, Immunology 1986.

Contents:

Introduction	5
aims	5
history	6
choice of macromolecular markers - methodological aspects	6
 Transmission of heterologous macromolecules	9
sources and possible routes	9
factors influencing	14
 Immunological implications	15
general comments	15
the local immune system	16
oral tolerance	20
maternal antibodies	23
immunological "education"	25
 Summary	30
 Acknowledgements	32
 References	33

Introduction

Aims

The multiple features characterising the phenomenon known as macromolecular transmission and the immunological implications occurring when heterologous macromolecules (HMM), here sometimes referred to as antigens, enter the organism are considered in this thesis. It will mainly deal with transmission of protein molecules from the small intestine into the blood during the perinatal period.

It seems to be a rule in all mammalian species studied so far that macromolecules (MM) with retained antigenicity are absorbed and transmitted over the gut much more readily in immature i.e. fetal and neonatal individuals than in adults. In this thesis I will try to explain the importance of macromolecular transmission (MMT) into the developing organism including both transfer of immunoglobulins from the mother and antigens from the environment. I will also discuss the possible "education" of the immunesystem by antigens and maternal antibodies present early during fetal and suckling stages. To simplify comprehension I would like to split the concept of MMT into two major parts although they are highly inter-related and often occurs simultaneously: a/ the possible fetal and neonatal contact with environmental macromolecules other than those of maternal origin. b/ The transfer of immunity (immunoglobulins) from mother to off-spring.

The original papers included in this thesis deal mainly with the basic mechanisms of macromolecular uptake from the intestine and the appearance of heterologous macromolecules in various body fluids (paper I-IV) and with an immunological application in Guinea pigs (paper V). The fetal and neonatal period and the pregnancy and lactation period of the adult are especially emphasised. The main part of the experimental work has been made using the rat (paper I-IV) and the guinea pig (paper V) as experimental animals, but the summary will also include comparisons with other mammalian species such as mice, rabbits, ungulates and humans. The summary is deliberately extended beyond the scope of the actual works presented but is an attempt to help the reader understand the subject in a wider sense.

History

From an historical point of view indirect evidences for uptake of macromolecules from food, was allready discovered and described in man by Hippocrates who observed that cow's milk proteins could cause gastric upset and utricaria. At that time adverse reactions to food was probably quite common but the exact mechanisms behind the illness was unknown and the symptoms were probably often mixed up with those of food poisoning and other gastrointestinal disorders. It was not until 1860 that more systematic studies of food hypersensitivity in man was made by Salter, and in the beginning of this century, animal experiments revealed that protein contained in the food was the cause of the sometimes very dramatic and even lethal reactions. The fact that it was necessary for undegraded proteins to enter the organism to produce these reactions brought about more extensive research into the uptake and transmission of heterologous macromolecules through various epithels and the immunological consequences of this phenomenon. Another field of research that naturally converged into this field was the study of transmission of antibodies from mother to offspring first described by Erlich (1892) in mice. It was later discovered that this phenomenon occured in many other species and was extensively reviewed by Brambell (1970).

Choice of macromolecular markers for studying transmission - some methodological aspects

Definitions:

Since there are no objective criteria for describing a macromolecule (MM), the MM will be defined on the basis of how it is handled by the absorptive cell. Molecules of approximately 1000 Dalton and larger seem to act as MM and are normally not able to pass the small intestinal epithelial cell barrier by diffusion, but has to be actively endocytosed to enter and could therefore be defined as macromolecules (Weström et al 1984ab, Weström et al 1986a). This is, however, a broad estimation and there are differences between different kinds of molecules, as will be discussed below.

Transmission markers:

At present there are two major types of MM:s used for studying transmission into blood:

- a. Polypeptides and proteins
- b. Polymers

Proteins of both homologous and heterologous origin are used for this purpose and two different methods are mainly in use, one using unlabelled and the other using labelled protein markers. When unlabelled proteins (paper I-IV) are used an immunological method is normally used for the detection unless the marker possesses an intrinsic biological activity, e.g. is an enzyme or a hormone which can be measured directly (Danforth 1959, Kidron et al 1982). Several immunological methods can be used for detection and some of the most common quantitative assays are listed below together with their approximate detection limits.

- Single radial immunodiffusion (Fahey & McKelvey 1965) 5µg/ml (paper I-IV)
- Electroimmunoassay or rocket electrophoresis (Laurell 1966) 1µg/ml (paper I-IV)
- Enzyme-linked immunosorbent assay (Engvall 1980) 5ng/ml
- Radioimmunoassay (Thorell & Larson 1978) 1ng/ml (paper IV)

All these methods recognize the protein molecule immunologically by the use of specific antibodies and this implies that the transmitted molecule is intact or large enough to be recognized by the antibody. These methods are often used for studying naturally occurring HMM:s in the organism from food for example, as well as in experimental studies where immunological consequences due to transmission of intact proteins are of interest. The major disadvantage with these methods is the possibility for cross-reaction of the "specific" antibody with other proteins present in serum. Another drawback is caused by the difficulties in using a protein already present in high concentration in serum e.g. using a homologous protein as a marker since the small amounts normally transmitted become indistinguishable from the amount already present. The use of labelled proteins is preferred by some investigators and the most common label is a radioactive isotope such as I^{125} , but also fluorochrome label such as fluorescein-isothiocyanate (FITC) has been used. Labelling the marker have certain advantages such as quick and easy assay and the advantage of using any pro-

tein which is possible to label. However, a great disadvantage is that the label can be disconnected from the marker, due to degradation in the gastrointestinal tract, and then taken up in free form. The free label can then disturb the assay, despite the use of TCA precipitation, by binding to other proteins present in serum (Skogh 1982).

In general the use of proteins as macromolecular markers is the most physiological approach to models for studying antigen uptake and transmission in the gut in various hypersensitivity reactions and transmission of immunoglobulins from mother to offspring.

Polysaccharides such as dextrans and polyethylene-glycols (PEG) are frequently used as MM markers. Their main advantages are their low toxicity, their resistance to degradation in most biological systems and the possibility of manufacturing them in a complete spectrum of different molecular sizes. PEG:s up to a molecular weight of 1000 Daltons can be used with high accuracy but larger PEG's are difficult to analyse with retained resolution between the different sizes (Chadwick et al 1977ab, Tagesson et al 1981). Since only the PEG:s larger than 1000 Daltons seem to behave as macromolecules (Weström et al 1986b) their use in these systems are hitherto limited. However PEG's with a molecular weight of 4000 have recently been used for transmission studies in rabbits (Seidman et al 1986). Dextrans labeled with fluorescein-isothiocyanate (FITC-D) are available in different sizes from 3000 to 160,000 and can be used as a MM markers. Despite their resistance to degradation, the serum concentration of FITC-D 70,000 in 14 days old rats is more than five times less than that of the similar sized protein BSA six hours after feeding equal amounts of the two markers (paper II). This indicates a fundamental difference in the handling of the two MM:s probably due to their different physiochemical properties.

Uptake and/or endocytosis markers:

The uptake of macromolecules into the enterocytes has been studied by various histological methods and the markers used are briefly listed below.

Proteins:

- Ferritin, electron dense visible in EM (Blochman & Winborn 1966)
- Horse radish peroxidase (HRP), product visible in LM. (Orlic & Lev

1973)

- HRP labelled proteins, product visible in LM (Chin-tarnglin 1980)
- Isotope labelled, making visible using autoradiography in EM and LM. (Wild et al 1972)
- Fluorescein or rhodamine labelled markers, visible in UVM.
- Gold labelled, visible in EM and LM (Hanssen & Sembara 1984).
- Unlabelled proteins, detected by immunohistochemistry and visualised by using an antibody labelled with any of the labels mentioned above.

Others:

- FITC-D, visible in UVM (Ekström et al 1986).
- Colloidal gold, visible in EM and LM (Hanssen & Sembara 1984).
- Bacterias, viruses and latex particles, for LM, (Sanders & Ashworth 1961, Schatten 1954, Worthington & Graney 1973, Mobbs & McMillan 1981).

Histological studies of uptake can also be performed along with the simultaneous estimation of transmission into bloodserum by combining the techniques mentioned above. This has been shown to be a useful method for comparative studies of the cessation of MM transmission, "closure", in the pig and the rat (Ekström et al 1986).

Transmission of heterologous macromolecules

Sources and possible routes

Origin of Macromolecules:

The vast majority of heterologous macromolecules (HMM) that the organism comes into contact with emanate from the food or from the microbiological flora of the gut. The gastrointestinal (GI) tract is therefore the most important site for entry of macromolecules into the body.

Weaning is the natural event where the food changes from milk which contains mainly homologous molecules, to a food of purely heterologous origin. This period is quantitatively speaking the most vulnerable for HMM to enter the body and the period where immunological reactions to food proteins i.e. hypersensitivities are very common. However, as

discussed below, there are possibilities for earlier contact with HMM.

Age dependence:

By studying the transfer of immunoglobulins (Ig) from mother to offspring it has been shown that in many species, like ungulates and rodents, there is also an abundant transmission of MM other than Ig:s during the neonatal period (paper I-III) (Brambell 1970, Kraehnbulh et al 1979). All species studied so far seem to have an elevated transmission of MM through the gut early in development which is gradually reduced with growing age (Fig 1, Paper III-IV) (Danforth & Moore 1959, Korenblat et al 1968, Worthington et al 1974, Warshaw et al 1974, Seifert 1976, Udall et al 1981, Robertson et al 1982, Vucavic 1983, Jacobsson et al 1986). The possibility of contact with HMM in the fetal and pre-weaning animal is therefore of great interest.

Prenatal sources and routes:

In the studies presented in this thesis it has been shown that after a single feed of marker proteins (MP) to pregnant rats (paper II) we were able to detect these proteins in the amniotic fluid and in the serum of the fetuses. Preliminary studies also show that after feeding pregnant guinea pigs we were able to detect immuno-precipitable amounts of the fed protein in the amniotic fluid. In other experiments on guinea pigs (data shown in Fig 1) we have shown that the fetuses easily transmit MP:s present in the amniotic fluid and high serum levels of MP:s are found. The major route for this transmission is probably from swallowed amniotic fluid via the small intestine into the blood although other possible routes such as via the lungs might also contribute. These results are supported by studies made on rats and monkeys (Orlic & Lev 1973, Colony & Neutra 1985, Lev & Orlic 1973) which show the presence of a marker protein, injected into the amniotic cavity, in intracellular vacuoles along the small intestinal epithelium of the fetus. The route for the MP's found in the rat fetuses, as described in paper III, is more obscure. The fed MP:s in this experiment was also found in the serum of the dam so the possibility of placental transfer must be considered.

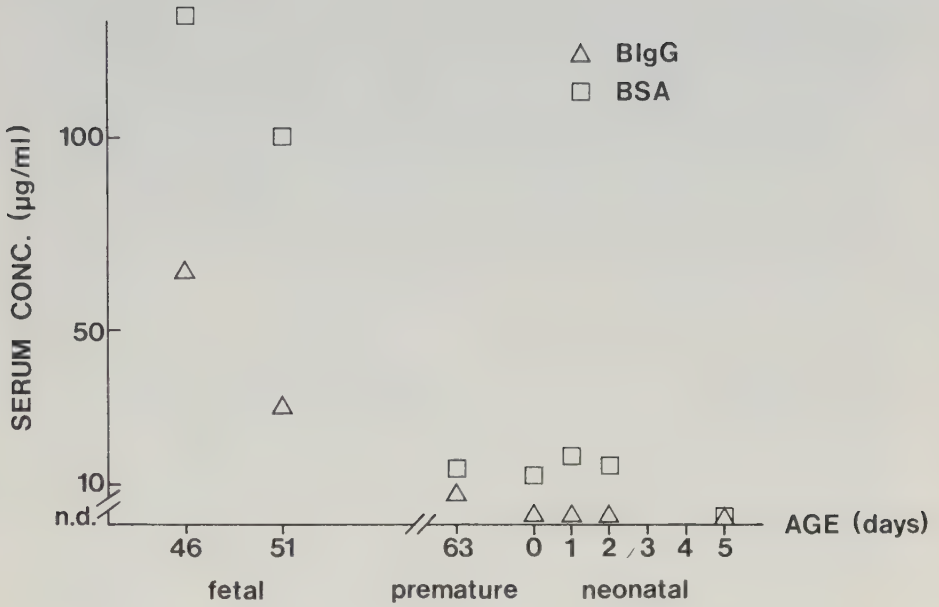


Figure 1.

The serum concentration of bovine IgG (BIgG) and bovine serum albumin (BSA) in fetal, premature and neonatal guinea pigs after feeding. The fetuses were "fed" by injecting a marker protein solution (bovine serum) 0.5 ml diluted 1/30 into the amniotic cavity and the fetal blood samples were collected 12 h later. Prematures and neonates were fed bovine serum by stomach tube 4ml/100g b.w. and blood samples were collected 4 h later.

Postnatal sources and routes:

Heterologous MM emanating from fed MP:s or the natural food have been detected in the milk of rats (paper IV), sows (to be published) and humans (Axelsson et al 1986). The amounts found are normally in the ng/ml range but exceeds often the serum concentrations estimated simultaneously. In the later part of the lactating period of rodents, pigs and other mammals another important source of HMM is bits of food and various other matter nibbled on in the surroundings.

Extent of transmission:

The transmission of MM's in neonates is a well documented phenomenon although most of the experiments were primarily designed for studying the transmission of immunoglobulins. With the exception of rodents, where the transmission of both heterologous and homologous IgG is greatly facilitated by the Fc-receptor present on the epithelial cells lining the ileum (Wallace & Rees 1980), IgG acts similarly to other macromolecular protein markers. As seen in Fig 1 premature and newborn guinea pigs possess an elevated transmission of MM:s with no preference for IgG, and there is a decrease in the transmission with growing age. A similar pattern is also seen in human infants in a studies using homologous α -lactalbumin as a marker (Jacobsson et al 1986) or homologous IgA (Vucavic 1983). In ungulates there is a massive transmission of MM:s the first 24-36 h of life (Porter 1969, Weström et al 1984ab) then there is a rapid decline and only smaller amounts of MM:s similar to those seen in newborn guinea pigs and neonatal rats are transmitted. After weaning the transmission is normally very low in all species studied and can only be detected with very sensitive methods (paper II-IV) (Walker 1972a, Warshaw 1974, Williams & Hemmings 1978, Stokes et al 1983b, Vellenga et al 1985). With the exception of IgG neonatal rats transmit MM:s in the same range as premature and newborn guinea pigs throughout the suckling period and transmission is only occasionally detectable after weaning (paper II), however by the use of very sensitive methods a low level of transmission seem to be consistent even during adulcy (paper IV).

As discussed above the amounts of MM:s transmitted during the neonatal period vary considerably depending on the species and most studies use the serum concentration of the marker as an estimation of the

transmission. This makes it difficult to compare different macromolecules, as their ability to enter and persist in the blood stream can differ considerably depending on degree of homology, size, digestability, structural and chemical properties and susceptibility for elimination. In the comparison between rats, pigs and guinea pigs given below the markers bovine IgG (BIgG), bovine serum albumin (BSA) and FITC labelled dextran were used for studying transmission. Similar protocols and amounts of markers were used.

In the preclosure piglet virtually all MM:s present in the gut are effectively endocytosed and transmitted into the blood-stream (Pierce & Smith 1967, Porter 1969). There are no evident differences between immunoglobulins and other MM:s or between homologous or heterologous MM:s in their ability of crossing the intestinal epithelium (Carlsson et al 1980). The total amounts of MM:s reaching the circulation of the piglet from colostrum this first day of life are in the range of several grams. During this period mg amounts of marker molecules per ml of bloodserum are found a few hours after feeding but by feeding the same amount of markers post closure (>36 h) the serum levels occasionally reach μg levels and in the adult pig only ng amounts are found (Weström et al 1984, Weström 1986, Telemo to be published).

In the preclosure rat, a considerable amount of both homologous and heterologous IgG is transmitted greatly exceeding the amounts of other MM:s transmitted (paper I and II)(Jones 1974, Morris 1975, Morris & Morris 1976). From a given dose of markers (BIgG, BSA and FITC-D 70,000) fed to 14 days old rats, about 3% of the IgG, 0.2 % of the BSA and 0.02 % of FITC-D were found in the blood serum 6 h later (paper II). In the postclosure (>21 d) and adult rat the serum levels found are generally in the ng/ml range but occasionally reaching μg levels (paper III-IV). In the prenatal and neonatal guinea pig up to 2 days of age, the transmission of MM:s is comparable with that found in the preclosure rat (except for IgG). At 5 days of age the transmission was undetectable with the methods used, i.e. the serum levels of the markers were less than 1 $\mu\text{g}/\text{ml}$ (Fig 1). In human prematures and newborns the serum levels after feeding sometimes reaches $\mu\text{g}/\text{ml}$ levels (Jacobsen et al 1986) but declines gradually with growing age and in adults only ng/ml levels are usually detected (Axelsson et al 1986). It should be noted that the comparison of the transmission in the different species and developmental stages is very inexact but is included as a rough guide for later discussions.

Factors influencing.

There are two main factors influencing the transmission of MM:s in the gastrointestinal tract:

1. The endocytotic activity of and the transport through the enterocytes.
2. The intraluminal and intracellular proteolytic activity.

The neonatal pig and rat are suitable models for demonstrating this. As mentioned above there is a massive transmission of MM:s in the preclosure piglet. In histological sections of the small intestine, numerous vacuoles are present in the epithelial cells which contain endocytosed material from the luminal contents (Comline et al 1951; 1953). However, even after the cessation of the massive transmission heavily vacuolated cells are found in the epithelium and the abrupt closure seems to be produced by arrested transcellular transport. The amount of vacuolated cells containing endocytosed material declines, however, with growing age and at about three weeks of age they have disappeared (Clarke & Hardy 1971, Lecce 1973). It has been proposed that this is due to an exchange of the epithelial cells from a fetal to a more adult type (Smith & Peacock 1980). In the piglet the intraluminal proteolytic activity is effectively suppressed by protease inhibitors present in colostrum (Laskowski et al 1957). This facilitates the transmission of proteins such as immunoglobulins from colostrum (Baintner 1973, Carlsson et al 1980).

In neonatal rats, the endocytotic activity of the small intestinal epithelium is not as extensive as in the piglet but judging from the uptake of polyvinylpyrrolidone or FITC-D the uptake disappears at about three weeks of age (closure) (Clarke & Hardy 1969a, Ekström 1986). As shown in papers I and II, the proteolytic activity in the intestines is of importance for the amount of protein transmitted and transmission can be elevated in the neonatal rat both by ligating the pancreatic duct and by adding trypsin inhibitors.

Other ways of elevating the transmission through the gut are by adding detergents such as lysolecithin (Bolin 1982, Talbot et al 1984) which sometimes occur naturally in the food or by causing a gut damage via pathogens and their products (Bloch et al 1979, Reinhardt 1984, Magnusson et al 1985; 1986) or an immunological reaction (Blockman & Winborn 1966, Kilshaw & Slade 1980, Strobel et al 1986). It is possib-

le that MM:s use other routes for entering through the damaged epithelial surface and an elevated "leakiness" rather than transport could explain some of these results.

Another factor that has been shown to modulate the uptake and transmission of a MM is an active immune response to the marker used either due to repeated use of a marker in the same individual or due to cross-reactivity between the marker and an environmental antigen. There are reports on both diminished uptake and enhanced elimination of a fed marker due to an active immune response to the marker used (Heremans & Bazin 1971, André et al 1974, Walker et al 1975, Seifert et al 1977, Swarbrick et al 1979, Walker 1986).

Conclusions:

It is clear that heterologous macromolecules emanating from the food of the mother can reach the fetus and the suckling offspring via the mother. It is also shown that during fetal and neonatal periods the transmission of MM is generally elevated as compared to adulthood.

These phenomena have been shown to cause immunological adverse reactions such as infantile colic in humans (Jakobsson & Lindberg 1978) and might be important in hypersensitivity reactions seen in early-weaned neonates. Such reactions are, however, rare and should be seen as the exception rather than the rule. In the following part an immunological interpretation of the role of MM entering the developing organism will be given.

Immunological implications

General comments

The major part of the immunesystem is located close to parts of the body exposed to the environment. The intestinal surface is by far the greatest surface area of the body exposed to the environment, but the respiratory tract, the skin, the ears and the eyes also contribute. Antigens penetrating any one of these surfaces comes in contact with a specialised mucosal immune system operating for the local defence at all these sites. The lymphoid tissue involved in the defence of the gastrointestinal tract is called gut associated lymphoid tissue (GALT)

and the respiratory equivalent bronchial associated lymphoid tissue (BALT). Work is in progress to define a skin associated lymphoid tissue (SALT) (Bos & Kaspenberg 1986). There is a high degree of communication between the different systems involving traffic of both regulatory and effector cells of the immunesystem (Rudzik et al 1975, McDermott & Bienenstock 1979) and it is postulated that they are all branches of a common mucosal immune system (Bienenstock et al 1979). The gastrointestinal (GI) tract is permanently exposed to enormous amounts of antigens. Besides the antigens provided by the normal intestinal flora, the highly variable food contains a great diversity of antigenic material (mainly proteins) and sometimes significant amounts of lectins and other nonspecific stimulators of the immune system. In addition, the food is a source of pathogenic microorganisms to which the presence of an effective defence system is vital.

A very important task for the immune system is to discriminate between potentially harmful antigens and all other antigens such as food proteins which are of no threat to the organism. The importance of having a functioning immune system in this sense is seen in some pathological conditions involving hypersensitivity reactions to various food proteins causing severe damage to the organism (Hemmings 1981)

The local immune system

"First line of defense":

The major role of the mucosal immunesystem seems to be to prevent microorganisms their toxic products and other toxic molecules (such as lectins) from entering the organism. There are thus some special features of the local immune system which make it differ from the systemic immunesystem. The major immunoglobulin class found in the GI tract in most mammalian species studied is IgA (Walker 1986). Ruminants are an exception to this rule and IgG₁ predominating (Morgan et al 1981). The majority (80%) of the antibody producing plasma cells (B-cells) lining the lamina propria of the entire intestine produce secretory IgA (sIgA) but about 20% are IgM and about 2% are IgE producing. IgG producing cells are also found but under normal conditions they are relatively few as compared to the amount of cells with the other three class specificities (Kagnoff 1981). The sIgA produced by

these plasma cells is transported through the epithelial cells by a specific transport system out into the intestinal lumen where it is mainly situated in the mucus coat overlying the microvillous surface (Tomasi & Grey 1972). The major roles of the IgA are to impair attachment of microorganisms to the epithelial surface by binding and covering their surface receptors and excluding antigens by trapping them in the mucus layer (Fubara & Freter 1972, Walker et al 1972b; 1975, André et al 1974, Stokes et al 1975, Swarbrick et al 1979, Lim & Rowley 1982, Newby et al 1982), thus preventing them from binding and entering the epithelial cells and also make them more susceptible for degradation by intraluminal enzymes (Walker et al 1975, Pang et al 1981). The sIgA itself is relatively resistant to proteolytic degradation. The large amount of sIgA (1-2 g produced daily in humans) that covers the intestinal surface makes up the "first line of defense" and is sometimes described as an "immunological paint". The IgA antibody seems to be an antibody made especially for exclusion since it does not activate complement and there is no evidences for opsonization or ADCC reactions mediated by IgA (Kagnoff 1981). These features are advantageous in the first line defence since IgA behaves as a "silent" immunoglobulin causing no damage while acting.

Apart from the IgA, system the defense of the GI tract have much in common with the rest of the immune system of the organism and shares the same effector mechanisms. Besides the antibody producing cells, the lamina propria region also contains T- cells of various types (discussed later), macrophages, eosinophils, neutrophils and basophils. In addition, intraepithelial lymphocytes (IEL) are present in high frequency (approx. 1/10 epithelial cells) just below the surface and very close to the epithelial cells (Meader & Landers 1967 Cerf-Bensussan et al 1983). The IEL:s express T-cell markers and their exact function is poorly understood but a possible cytotoxic (Mowat & Ferguson 1982, Ernst et al 1986) and/or supressor function has been suggested (Lyscom & Brueton 1982, Klein 1986).

Along the intestine there are certain macroscopically visuable areas or "islands", with high numbers of immune cells, mainly B-cells (Craig & Cebra 1971), called Peyer's patches. The epithelial surface overlying these areas consists of a high percentage (approx. 15 %) of specialised epithelial cell called membranous cells or M-cells (Wolf & Bye 1984, Egberts et al 1985). The M-cells differ morphologically from

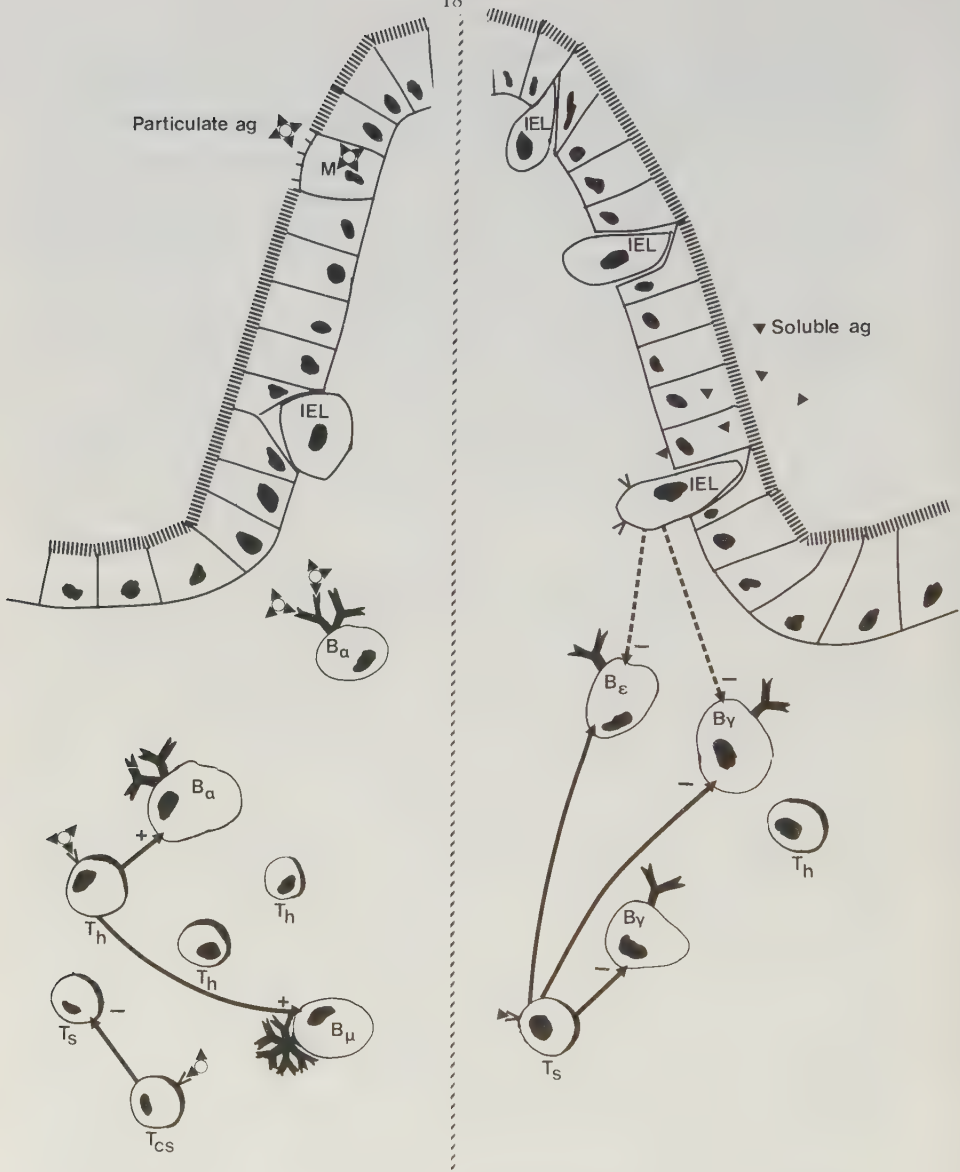


Figure 2.

Possible model for regulation of the immune response to a replicating particulate antigen (left) and to a non-replicating soluble antigen (right).

B_α = B-cell producing IgA

B_μ = " " IgM

B_γ = " " IgG

M = M-cell

T_S = T-suppressor cell

T_h = T-helper "

T_{CS} = T-contra-suppressor cell

IEL = intra epithelial lymphocyte

normal epithelial cells and show a paucity of microvilli, a poorly developed glycocalyx and absence of lysosomal organelles. M-cells seem to be adapted for antigen transport and have been shown to be active in phagocytosis and pinocytosis of material present in the lumen for a direct presentation to the lymphoid cells concentrated in this area. It is, however, uncertain how much these cells contribute to the total transport of antigens across the GI epithelium and if there is any qualitative difference in the handling of antigens presented by the two cell types (Fig 2). However, since the normal epithelial cells have been shown to express class II (Ia) antigens (Barclay & Mason 1982, Mayrhofer et al 1983) and present antigens (Bland & Warren 1986a) a different role of the two cell types might be proposed. It is possible that the M-cell mainly deals with particulate antigens and are normally involved in the production of an effector response. In this model the main role for the normal epithelial cells could be to present soluble antigens to the IEL:s thus maintaining the tolerance to "harmless" antigens or produce cytotoxic reactions to "harmfull" antigens depending on prior experience. In in vitro experiments antigen presenting epithelial cells have been shown to preferentially stimulate T_S -cells (Bland & Warren 1986b).

The lymphocytes stimulated in the Peyer's patches have been shown to leave the patches and via the blood and/or the lymphatics, to enter the spleen and/or the mesenteric lymph nodes for maturation and then return to the intestine to colonize the lamina propria region and produce an active immune response (Weiz-Carrington et al 1979).

T-cell function:

Regulatory T-cells are crucial for regulating the immune response of the great number of antigens entering via the GI tract (Fig 2). Cells expressing the T lymphocyte markers are numerous in the whole mucosa and in the lamina propria where they account for 35-90 % of the total amount of the lymphoid cells present depending on the species studied. The two main types of regulatory T-cells are T-helpers (T_h) and T-suppressors (T_S). An additional T-cell subset called T-contrasuppressors has recently been described (Gershon et al 1981, Suzuki et al 1986). These cells act by antigen-specific suppression of the T_S abrogating their suppressive effect on the T_h which gives a net result of an enhanced immune response to the antigen (Fig 2). A

detailed description of the complex regulation by the T-cells is beyond the scope of this thesis but some characteristics of the regulatory cells will be given. T_h cells enhance the immune response to replicating antigens presented by macrophages or other antigen-presenting cells and are also stimulated nonspecifically by bacterial endotoxins (Hamoaka & Katz 1973, Uchiyama & Jacobs 1978) and inflammatory substances produced by granulocytes. T_s cells are common in Peyer's patches but T_h cells are dominating in the lamina propria (Kagnoff 1981). There are some confusion regarding the function of the IEL:s as a great proportion of them express the supressor markers. T_s play an important role in the down-regulation of the response to non-replicating antigen such as food proteins (discussed later) that enter the organism from the intestine (Fig 2). A dual action for the T_s resulting in suppression of the IgG and IgM classes and enhancement of IgA has also been proposed (Elson et al 1979, Richman et al 1981). Evidence for an antigen specific contra suppressor cell favouring IgA response have recently been reported (Fig 2)(Suzuki et al 1986).

Oral tolerance

It has been shown that oral presentation of a variety of protein antigens (Richman et al 1978, Swarbrick et al 1979, Heppel & Kilshaw 1982, Stokes 1983ab)(paper V) and sheep red blood cells (Kagnoff 1978ab; 1980) diminish or completely suppress the subsequent response to a parenteral immunization with the antigen even when strong adjuvants are used (Thomas & Parrot 1974, Hanson et al 1977, Tomasi 1980). The suppression involves both systemic antibody production and cell mediated delayed type of hypersensitivity (DTH) response (Kagnoff 1978a, Mowat et al 1983, Strobel & Ferguson 1986). The induction of tolerance can be abrogated by pretreatment with cyclophosphamide (Mowat et al 1982, Strobel et al 1983) and can be transferred to a recipient by adoptive cell transfer of Peyer's patch or splenic lymphocytes (Ngan & Kind 1978). These results and those from others (Asherson et al 1977, Richman et al 1978, Hanson et al 1979, Richman 1979, Titus & Chiller 1981) suggest that oral tolerance is an active state maintained by antigen specific T_s -cells (Fig 2). Under some circumstances tolerance has also been shown to be transferable to a recipient by soluble serum factors (André et al 1975, Chalon 1979, Kagnoff 1978b 1980, Mattingly et al 1980). A biphasic response to fed antigens with

an enhanced local IgA response and a suppressed systemic IgG response has also been suggested (Elson et al 1979, Challacombe & Tomasi 1980, Richman 1981). By prolonged feeding with an antigen, however, the IgA response also declines (Kagnoff 1982). Tolerance induced by excessive amounts of antigen injected i.v. or fed to neonatal rodents are believed to create a clonal deletion or paralysis of the B-cells (Dresser 1968, Pillai & Scott 1983). However, in the T_S -cell mediated tolerance the antigen specific B-cells have been shown to be primed but functionally suppressed by antigen specific T_S -cells (MacDonald 1983).

Feeding an antigen together with killed *Bordetella pertussis* cells (Hof et al 1975) or with a simultaneous i.p or i.v. injection of the adjuvant (Jarret et al 1976, Bazin & Platteau 1976, Mowat et al 1986) shifts the response to a sensitization to the antigen fed. This is probably due to an activation of the T_H cells by a non-specific stimulation produced by the bacterias which is the postulated role for any adjuvant used in immunization procedures.

In general, it seems to be difficult to produce persistent local or systemic immune response to any soluble antigen presented orally without using adjuvizing regimens like endotoxins, live or killed bacterias or virus or other particulate vectors (Challacombe & Tomasi 1980, Wachsmann et al 1985). However, when feeding the antigen, prior to the tolerant state there is often a phase of transient elevated responsiveness (Asherson et al 1977, Stokes et al 1983a, McDonald 1982) of both delayed type of hypersensitivity (DTH) (Kagnoff 1978a) and antibody response (Kagnoff 1978b).

Tolerance can be induced much more easily in neonatal animals than in adults (Filipp 1965, Halsey & Benjamin 1976, Benjamin 1977, Rieger et al 1981, Heppel & Kilshaw 1982)(paper V). However a certain degree of maturation of the organism and presumably the immune system, seems to be necessary for the induction of an active tolerant state, since feeding mice that are less than 7 days old did not result in tolerance and feeding before 2 days of age resulted in priming i.e. an elevated immune response to subsequent challenge (Hanson 1981). These results have later been confirmed and include both cell mediated immune response (CMI) and antibody response (Strobel & Ferguson 1984; 1986). Guinea pigs are more mature at birth than mice, and are easy to tole-

Table 1.

Time and route of transmission of humoral immunity

Animal order	Species	Transmission		Duration of postnatal transfer
		Prenatal	Postnatal	
Primates	Man	Hemochorial	-	
	Monkey	placenta		
Lagomorphs	Rabbit	Yolk sac	(Small intestine)	?
Rodents	Guinea pig	Yolk sac*		
	Rat	Yolk sac	Proximal intestine	21 days
	Mouse	Yolk sac	Proximal intestine	20 days
Carnivores	Cat	Placenta and/or	Gut	1-2 days
	Dog	yolk sac	Gut	1-2 days
Ungulates	Cow	-	Entire small intestine	24 h
	Goat	-	Entire small intestine	24 h
	Sheep	-	Entire small intestine	24 h
	Pig	-	Entire small intestine	24-30 h
	Horse	-	Entire small intestine	24 h

*Late in gestation probably also from the amniotic fluid via the intestine

size at birth (paper V) compared to later in life (Heppel & Kilshaw 1982). The results from paper V also suggest the possibility of tolerance induction already in utero. From about two months of age Guinea pigs are difficult to tolerize and feeding a protein antigen to adult animals has been shown to produce sometimes fatal anaphylactic reactions (Devey et al 1976, Anderson et al 1979). However a prolonged feeding of an antigen can result in a refractoriness to these reactions (Coombs et al 1978) but it is not shown if there was active suppression of the response following parenteral challenge.

Conclusions:

It seems that oral tolerance is a basic immunological mechanism and is normally induced by soluble, nonreplicating antigens fed to immunologically mature animals provided that no infection or other adjuvizing factors act simultaneously. The tolerant state includes both suppression of CMI and antibody response specific to the antigen and is probably important for avoiding defence actions against "harmless" antigens entering the organism. There are numerous examples of pathological conditions which could be related to a disturbed or disrupted tolerant state such as hypersensitivity reactions, food enteropathies, atherosclerosis, rheumatic disorders and autoimmune conditions.

Maternal antibodies

Transmission to the offspring:

Transfer of immunoglobulins from mother to young is a general phenomenon occurring in all mammalian species studied so far (Brambell 1970, Kraenbuhl et al 1979) and has also been described in the fetal chick (Brierly & Hemmings 1956). It is mainly IgG of the subclass IgG1 that is transferred in significant amounts. There are three possible routes for transmission:

1. via the yolk sac
2. " the small intestine
3. " the placenta

The species differences hitherto reported are summarised in Tab 1. The transmission of IgG is very effective and when terminated the serum concentration in the offspring equals and sometimes exceeds adult levels (Curtis & Bourne 1970, Hyvarinen et al 1973). Specialised

transport mechanisms have evolved to facilitate transmission, often with a high degree of selectivity. Since the IgG transmitted emanates from the maternal circulation trans-cellular transport occurs in the yolk sac, placenta and/or mammary gland according to the species studied (Tab. 1). In the endodermal cells of the yolk sac splanchnopleur, syncytiotrophoblasts of the placenta (of fetal origin) and alveolar mammary, cells the transcellular transport of IgG is Fc-receptor mediated. The endocytosed receptor-bound IgG is contained in "coated vesicles" of a relatively small size and the term micropinocytosis is often used to describe their histological appearance (Wild 1972, Goldstein et al 1979). In rodents the postnatal transmission of IgG in the proximal small intestine is mediated by a similar mechanism (Rodewald 1973, Rodewald et al 1982) as discussed earlier and in papers I & II.

Colostrum and milk antibodies:

The most striking difference between the immunoglobulin content of colostrum and milk is seen in ungulates where the major antibody in ruminants is serumderived IgG₁ (Porter 1972) and in pigs serum derived IgG₂, present in high concentration (Bourne & Curtis 1973). This is the sole source of immunoglobulins for the newborn, since it is born with an almost total lack of immunoglobulins (Chaniago et al 1978) (Tab. 1). In milk from all species but ruminants and in colostrum and milk from primates the major immunoglobulin is secretory dimeric IgA. The IgA is produced locally in the mammary gland (Ahlstedt et al 1975) by plasma cells recruited from the GALT via the so-called enteromammary link (Roux et al 1977, Kleinman & Walker 1979). The exception seen for ruminants where IgG₁ dominates also in milk could be explained by the fact that IgG₁ is the dominating immunoglobulin in the enteric secretions in these species (Morgan et al 1981).

The antibodies in colostrum and milk provides an important local protection from pathogens in the GI tract (Wilson & Svendsen 1971, Scott et al 1972, Rutter & Jones 1973). The antibodies hereby "selects" the bacterial flora according to the same pattern found in the maternal intestine by means of the "silent" non-inflammatory action of IgA. The breast milk also contains other more or less specific factors for "directing" the bacterial flora such as; live lymphocytes and granulocytes, interferon, lysozyme, complement, bifidus factor, lactofer-

rin, lactose and others (for a review see Head 1977). The passive protection by the milk in the GI tract of the newborn creates a stable milieu for the terminal development of the local immune system during neonatal life as will be discussed in the following section.

Immunological "education"

The view that the function of maternally-derived antibodies has been solely as a passive protection of the offspring against pathogens in the environment, must now be widened. There is growing evidence for an immunoregulatory role for these passively acquired antibodies.

In 1974 Jerne presented his "network" hypothesis, which is based on the antigenicity of the hyper variable site (idiotope) on the immunoglobulin molecule and its capacity for eliciting an immune response within the individual. The first antibody produced by antigenic stimuli is called an idiotype (Id) the second antibody produced in response to the first antibody is called an anti-idiotypic antibody (aId) (Fig.3). As a result of this phenomenon an antigen introduced into the organism creates a network of idiotypic-antiidiotypic relations since both the Id and aId carries more than one idiotope. A network model has also been proposed for the regulatory T-cells (McNamara & Kohler 1984) which provides a possible explanations for the regulation and memory of the immune response. An interesting feature of some of the aId:s is their ability to mimic the appearance of the antigen and thus serve as an "internal image" of the antigen (Jerne et al 1982) (Fig.3). AId:s have been successfully used for producing a vigorous immune response to their corresponding antigens often exceeding the response of the antigen itself and their use as vaccines are currently being investigated (UytdeHaag et al 1986, Kennedy et al 1986).

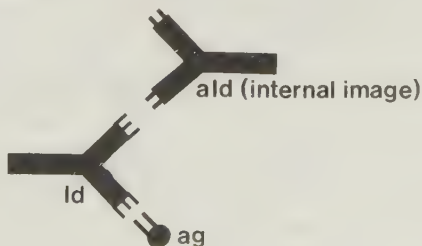


Figure 3.

An idiotypic antibody (Id) with its corresponding anti-idiotypic (aId) which in this case mimicks the antigen and thus serves as an "internal image".

The role of maternal antibodies:

"Challenge" of the fetus or newborn with the maternal idiotypes during development presumably cause some kind of immunological response. The immune system as studied in human fetuses is fully capable of eliciting an immune response including both CMI (Toivanen et al 1981) and later IgM antibody production from 10-15 weeks of gestation (Stiehm 1975, Gathings et al 1981). Other characteristics of the immature immunesystem is that T-cells from fetal blood, regardless of if they express helper or suppressor surface markers, suppress the mitogen response of adult lymphocytes in a mixed lymphocyte culture (MLC) (Andersson et al 1983) and all B-cells have IgM on their surface but some co-express IgG and IgA (Miyawaki et al 1981). The same pattern is seen in other mammalian species studied (Richardson & Conner 1972, Conner et al 1973; 1977) and indicates a certain degree of functional maturity of the immune system with the ability to respond to antigenic stimuli. It is therefore likely that stimuli from maternal idiotypes produce an IgM response in the offspring. In adult mice passively transferred antigen-specific IgM followed by injection of the antigen has been shown to greatly enhance the subsequent immune response to the antigen (Henry & Jerne 1968, Collisson et al 1979). Another interesting finding is that 75% of the T_h cells in adult peripheral blood carry the Fc_μ receptor (Andersson et al 1981). In theory this provides the possibility to activate anti-idiotypic T and B-cells with an IgM idotype coated T_h cell. The anti-idotype thus produced could be looked upon as an internal image of the antigen which has the possibility of activating idotype-specific T_h and B-cells (Rubinstein et al 1982) i.e. expanding the clones originally stimulated without any contact with the "true" antigen. Later exposure to the "true" antigen would then produce a rapid onset of the immune response due to the priming effect of the maternal aid.

There are some interesting experimental findings supporting this theory. Newborn mice injected i.p. with a small amount (50 ng) of a monoclonal IgG1 aid produced in response to an IgM idotype directed against a pathogen (E.coli K13) showed a dramatic change in resistance to a subsequent challenge with a 30xLD50 challenge of the live E.coli (Stein & Söderström 1984). The survival rate was about 80 % compared to 0 % in the controls. The same effect was achieved in litters where the dam had been injected at delivery with 10 µg of the aid. Adminis-

tering the idiotype according to the same protocol also gave protection but to a lesser extent and the effect not as long lasting. A certain protection (33 %) was also seen when the antigen itself was injected into the newborn. In this system and in others (Rubinstein et al 1982) it has been shown that the dose of the subclass of aId administered is of great importance for the response achieved i.e. high doses (1-10 µg) produce less priming or even tolerance and aId of the IgG₁ subclass produces priming while that of IgG₂ produces suppression. It should also be noted that IgG₁ is the subclass preferentially transferred from the mother and that the amounts of aId experimentally found to be priming is similar to the amounts occurring naturally in the organism (Roitt 1985).

The very dose restricted response seen especially when using neonatal animals could be due to the the very few idiotope specific B- and T-cells present in the non-stimulated organism. The extremely small amounts (10-100 ng) of aId needed to produce priming shows the very high potential and specificity of the system.

Other experiments where maternal antibody responses seem to modulate the immune response of the offspring have been done in rabbits (Jarret 1983) where the offspring to OVA immunized dams showed an elevated IgG response to OVA, but interestingly a diminished IgE response. Priming has also been shown in guinea pigs where the offspring of an OVA immunized mother showed a vigorous response to a low dose of antigen normally used for boosting, a dose that does not produce any response in a normal guinea pig born by a nonimmunised dam. (Andersson et al 1985). In this experiment it is possible that small amounts of maternal idiotype specific antibodies remained in the serum of the offspring, facilitating the presentation of the injected antigen to the immune system. However, since the response was consistent in a third generation of Guinea pigs, without any challenge of the intermediate generation, this explanation does not seem very likely.

In calves the response to fed soya protein is enhanced by maternal anti-soya antibodies (Barrat & Porter 1979). Elevated response to vaccination was seen in infants born by mothers immunized against pertussis-diphtheria-tetanus (Levi et al 1969) and tetanus toxoid (Gill et al 1983) and similar findings have also been reported in pigs (Hoerlein 1957, Segre & Kaberle 1962a). However, a common phenomenon seen is a

transient antigen-specific hyporesponsiveness in the offspring of hyperimmunized mothers which could be due to high titres of maternally derived idiotypic antibodies "protecting" the offspring (Segre & Kaberle 1962b, Izuchi et al 1984). In general there is a delayed and poor or even abrogated immune response to a range of pathogen-derived antigens in neonatals deprived of maternal antibodies. This could be due to a lack of "antigenic" stimulation from maternal idiotypes and thus a poorly developed immune system.

Environmental antigens presented via the mother:

As shown in paper III & IV, protein antigens fed to pregnant and lactating rats are found in the fetal circulation and in the milk in an antigenic, apparently unchanged form. Dietary antigens have also been detected in human milk (Jakobsson et al 1985). In an attempt to evaluate the immunological consequences of this phenomenon an experiment was performed according to the following protocol (to be published). Female rat pups were fed a milk whey protein solution (the same as that in paper V) by stomach tube from their 7:th day of age. From weaning (at 3 w of age) they were continuously fed a diet containing milk proteins. At 3 month of age they were mated. Within two days after delivery pups from control dams on a milkfree diet were cross-fostered onto the milk fed dams (together with their own pups). This "mixed litter" then continued on the milk-containing diet from weaning and at 10 w of age they were all immunized with milk proteins using alum as adjuvant. The results showed an almost total lack of response (IgG titre) in the cross-fostered rats and a large response, exceeding that of immunized control rats on a milk-free diet, in the rats born by the milk protein fed dam. In an additional experiment, the same protocol was followed with the only difference being that the dam was fed a milk-free diet from mating until delivery i.e. during pregnancy. This experiment produced principally the same results. This was a somewhat unexpected finding but demonstrates clearly the influence of maternal antigen experience on the subsequent response to the antigen in their offspring. It indicates that priming takes place early in development mainly during fetal life since the milk of these dams did not prime the cross-fostered pups. It also indicates that the priming of the offspring was not due to the presence of the antigen during pregnancy. It is possible, although unfortunately not tested, that the dams were sensitized to the milk proteins, due to the early feeding of

large amounts of milk proteins, rather than tolerized. If so, the priming could be due to maternal antibodies.

The role of the stage of maturity for the response to an antigen is (as discussed earlier) clearly shown in mice (Hanson 1981, Strobel & Ferguson 1984) supports some of the results mentioned above. Another interesting observation made in mice is that the IEL begin to populate the intestine at 5-10 days of age (Ferguson & Parrott 1972, Shields & Parrott 1985). These data suggest that there is a demand for a certain maturation of the immune system in rodents before an active state of tolerance can be induced. Moreover, it is likely that antigens contained in the milk tolerize the pup provided that the dam has not reacted to the antigen.

Guinea pigs and rabbits are easily tolerized by feeding protein antigens from the first day of life (paper V) (Filipp 1965; 1967, Udall et al 1984). In addition the results of paper V shows that induction of tolerance can occur already in utero. Also in this experiment, however, circumstantial evidences suggest that differences in maternal experience of the antigen can produce different responses in the offspring. Rabbits born and nursed by soya protein fed mothers have been shown to become tolerant to soya protein via the mother (Patriana et al 1981). The immunological role of dietary antigens presented via the dam during pregnancy and lactation is still obscure, but a lack of response in the dam might promote tolerance in the offspring in a dual way by a/ elevated uptake and diminished elimination of the antigen in the dam facilitates presentation to the offspring (Benjamin 1977, Kleinmann et al 1983). b/ lack of priming antibodies.

Another observation that could be of relevance is that "biologically filtered" antigens i.e. fed antigens that have passed the GI tract into the circulation induce tolerance more easily than when injected (Bruce & Ferguson 1986). On the other hand, in the actively responding dam protection of the offspring from tolerizing contact with the antigen can occur (Kleinmann et al 1983, Harmatz et al 1986) and aid in the presence of antigens and/or immune complexes might facilitate priming of the offspring both pre- and postnatally.

Finally caution must be taken in the interpretation of studies

were immunoglobulins are used as antigens or hapten carriers, especially in newborn rodents, since the Fc part of the molecule might alter handling by the immune system, possibly by mimicking maternal antibodies. A common phenomenon seen in tolerance experiments on neonatal mice using relatively high amounts of IgG's as antigens or carriers is B-cell tolerance or "paralysis" (as discussed earlier) devoid of specific T-suppressors (Kettman et al 1979, Waters & Diener 1983).

Conclusions:

It is evident that both environmental antigens and maternal antibodies are present during the development of the immune system of the organism. This provides the possibility for an immunological "education" of the offspring including both tolerance and priming. It is speculated that maternal immunological experience can be transferred by an "extra genetic" mechanism producing an active immunological state in the offspring, thus preparing the organism for a proper response to environmental antigenic challenge. Under normal circumstances this must be of great benefit for the survival of the individual but may also provide the possibility for "inheriting" immunological "errors" such as hypersensitivity and autoimmune diseases.

This mechanism should also be considered in immunological experiments made on animal species born or provided with maternally derived antibodies or antigens as their immunological "virginity" may be questioned.

Summary

Intestinal macromolecular transmission of protein markers was studied in the neonatal rat and in the pregnant and lactating dam. Macromolecular transmission into the blood stream with retained immunogenicity was shown to be elevated in the neonate until 3 weeks of age but declined rapidly thereafter. During adulthood small amounts of fed markers are found in serum, amniotic fluid and milk of the pregnant and lactating rat. Heterologous macromolecules emanating from the food of the mother can thus reach the fetus and the suckling offspring via the mother. Soluble antigens presented orally during the neonatal

period of different species have been shown to produce immunological hyporesponsiveness (tolerance) more easily than in adulthood. In this thesis evidence is presented for induction of tolerance during fetal life to dietary antigens presented via the mother.

It is proposed that the main reason for the intestinal uptake of undegraded antigenic macromolecules of heterologous origin is to provide "information" from the surroundings on the present antigenic repertoire to the local and systemic immune system. This is necessary for producing and maintaining the active regulation which leads to a suppressed or enhanced immune response to an antigen.

It seems that oral tolerance is a basic immunological mechanism and is normally induced by soluble, nonreplicating antigens fed to immunologically mature animals provided that no infection or other adjuvizing factors act simultaneously i.e. during the suckling period. The tolerant state includes both suppression of CMI and antibody response specific to the antigen and is probably important for avoiding defence actions against "harmless" antigens entering the organism. There are numerous examples of pathological conditions which could be related to a disturbed or disrupted tolerant state such as hypersensitivity reactions, food enteropathies, atherosclerosis, rheumatic disorders and autoimmune conditions.

The role of maternal antibodies transferred to the offspring and their possible active priming via the different idiotypes of the immunoglobulins is also discussed. It is concluded that both environmental antigens and maternal antibodies are present during the development of the immune system of the organism. This provides the possibility for an immunological "education" of the offspring including both tolerance and priming. It is speculated that maternal immunological experience can be transferred by an "extra genetic" mechanism producing an active immunological state in the offspring, thus preparing the organism for a proper response to environmental antigenic challenge. This mechanism should also be considered in immunological experiment made on animal species born or provided with maternally-derived antibodies or antigens as their immunological "virginity" may be questioned.

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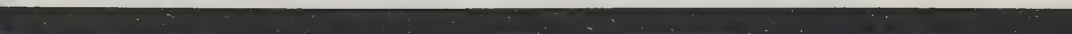
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Proteolytic Activity as a Regulator of the Transmission of Orally Fed Proteins from the Gut to the Blood Serum in the Suckling Rat

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Key Words. Protein absorption · Intestinal · Protease inhibitors · Proteolysis · IgG · Albumin · Neonatal rat · Serum

Abstract. 14-day-old rats were orally fed with porcine colostrum or serum having a low or high activity of protease inhibitors (i.e., sow colostrum trypsin-chymotrypsin inhibitor or soybean trypsin inhibitor). The uptake of undigested porcine albumin and IgG to the blood serum of these rats was studied 4 h after feeding. The effect of the exclusion of proteases to the intestinal lumen by means of pancreatic duct ligation prior to feeding was also studied. The results from these feeding experiments in the presence of protease inhibitors or in the absence of pancreatic proteases agreed well. It was concluded that for the suckling rat the intraluminal proteolytic activity in the gastrointestinal tract is a regulator for nonselective protein uptake, as represented by albumin, while the selective absorption of IgG was unaffected.

Introduction

The suckling rat has for some years served as a model for the study of the transmission of undergraded or partially degraded dietary proteins across the gut. There is a high degree of selection in this transport, favoring the transmission of immunoglobulins of the IgG class. The selective transmission is believed to be mediated via IgG receptors in the epithelial cells of the proximal small intestine [2, 22, 25]. In addition, proteins other than immunoglobulins have been shown to be absorbed undegraded [11, 14, 25], probably via a more nonselective basal endocytic route [cf. 29]. A number of factors affecting the

transmission of proteins have been suggested, but little is known about the role of gastrointestinal proteolytic activity. However, in the neonatal piglet, the specific trypsin-chymotrypsin inhibitor of sow colostrum has been observed to have a positive effect on the nonselective absorption of colostrum proteins [4, 28].

The present study investigated the influence of proteolytic activity in the intestines under physiological conditions on selective and nonselective protein transmission in the rat. The intestinal proteolytic activity was experimentally altered either by adding protease inhibitors to the orally fed protein solution, or by totally excluding proteases from

the lumen by means of pancreatic duct ligation prior to oral feeding. Porcine IgG and albumin were used as proteins for studying selective and nonselective transmission, respectively, in 14-day-old rats.

Materials and Methods

A total of 99 fourteen-day-old rats of the Sprague-Dawley strain were used in four separate experimental series. The young were separated from their dams, placed in heated cages (approx. 30 °C), and starved either for 4 h before feeding with sow colostrum, or starved for 8 h before feeding with porcine serum. The protein solutions used for feeding were given intragastrically at a dose of 0.05 ml/g body weight. The porcine serum used for feeding consisted of a serum pool obtained after the bleeding of pigs at slaughter. Sow colostrum, defatted by centrifugation, was obtained by the manual milking of several sows during farrowing, and then was pooled.

After feeding, the rats were kept separated from their dams for a further 4 h and were then killed. Blood samples were collected by heart puncture and were allowed to clot at room temperature. The blood was centrifuged at 3,000 g for 10 min and the serum was stored at -20 °C until analysis. The small intestine was removed from the carcass immediately after death and divided into one proximal and one distal portion of equal length. Each intestinal half was flushed with 1 ml ice-cold 0.15 M NaCl and the samples of intestinal contents were stored at -20 °C until analysis.

Procedures for Feeding Sow Colostrum

38 rats from four different litters were fed with sow colostrum. Within each litter, half of the animals were fed with sow colostrum having the normal high trypsin-inhibiting capacity (TIC), 7.8 IU/ml, while the littermates were fed sow colostrum to which 3 mg porcine trypsin/ml had been added, which is the amount just sufficient to eliminate the TIC [4]. The concentrations of albumin and IgG in both the untreated and treated sow colostrum were 17.0 and 45.0 mg/ml, respectively.

Procedures for Feeding Porcine Serum

36 rats from four litters were used in this experimental series. Within each litter, half of the young

were fed with untreated porcine serum having a TIC of 3.7 IU/ml, while their littermates were fed porcine serum to which had been added 2.0 mg/ml lyophilized soybean trypsin inhibitor (Sigma, St. Louis, Mo.), resulting in a TIC of 11.2 IU/ml. The concentration of albumin and IgG in both the untreated and treated porcine serum were 38.0 and 40.0 mg/ml, respectively.

Procedures for Feeding Porcine Serum after Ligation of the Pancreatic Duct

15 rats from two litters were separated from their dams 8 h before feeding. Within 1 h after separation, the young were anesthetized with sodium pentobarbital (50 mg/kg) and subjected to laparotomy. The pancreatic duct was ligated with a silk suture and the peritoneum and the skin were then separately closed with sutures. Sham operations (i.e., laparotomy without ligation of the pancreatic duct) were performed on 5 of these rats. All operations were carried out under semisterile conditions. The rats were allowed to recover in a heated cage (approx. 30 °C), and 8 h after separation from their dams they were fed with porcine serum as described above.

Procedures for Feeding Varied Concentrations of Porcine Serum

10 young from one litter were fed with varying dilutions of porcine serum. They were divided into five groups of 2 rats each, and fed with porcine serum, 0.05 ml/mg, in the concentration of 12.5, 25, 50, 100 or 200%. The dilutions were made using 0.15 M NaCl. The 200% serum solution was prepared by resolving freeze-dried porcine serum in distilled water.

Protein Analyses

The porcine albumin (P-alb) concentration in all samples was analyzed by electroimmunoassay [18] using a monospecific rabbit antiserum to P-alb [4]. Purified porcine albumin, fraction V (NBC, Cleveland, USA) was used as a standard. Porcine IgG (P-IgG) was quantitated by means of radial immunodiffusion [5], using monospecific rabbit anti-porcine IgG. Isolated P-IgG (Miles, Slough, UK) was used as a standard. Samples from each experiment were also analyzed with immunoelectrophoresis [23] to control the presence of protein fragments.

Determination of Trypsin-Inhibiting Capacity

The TIC of the feeding solution and the intestinal contents were determined spectrophotometrically us-

Table I. The mean concentration ($\mu\text{g/ml} \pm \text{SD}$) of porcine albumin (P-alb) and IgG (P-IgG) in serum, and the proximal and distal portions of the small intestine of 14-day-old rats 4 h after oral feeding with porcine colostrum, having a high trypsin-inhibiting capacity (TIC = 7.8 IU/ml), or porcine colostrum depleted of TIC by the addition of trypsin

	Num- ber of rats	Serum		Proximal small intestine		Distal small intestine	
		P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$
Colostrum	19	23 ± 6	253 ± 24	$2,106 \pm 204$	$6,184 \pm 583$	$1,273 \pm 413$	$3,584 \pm 773$
Colostrum + trypsin	19	$9 \pm 3^{***}$	$220 \pm 17^{***}$	t	t	t	t

t = Trace amounts.

*** Significance in comparison with colostrum-fed rats, $p < 0.001$ (Student's t test).

ing benzoyl-*DL*-arginine-*p*-nitroanilide as a substrate for trypsin [6]. One inhibiting unit (IU) corresponded to an inhibition of 0.4 mg porcine trypsin. Negative TIC values were taken as evidence for proteolytic activity.

Results

Effect of Feeding Sow Colostrum

The young rats in the group fed sow colostrum (TIC = 7.8 IU/ml) had an average P-alb concentration of $23 \mu\text{g/ml}$ in their blood sera 4 h after feeding, while the rats given inhibition-free colostrum showed a lower value of $9 \mu\text{g/ml}$ (table I). The serum concentration of P-IgG was $253 \mu\text{g/ml}$ in the group fed colostrum and somewhat lower, $220 \mu\text{g/ml}$, in the group fed inhibition-free colostrum. Immunoelectrophoresis revealed that in the group given inhibition-free colostrum, traces of partially degraded P-alb having a changed electrophoretic mobility could be observed in some serum samples. However, no breakdown products of P-IgG were found in these samples. The intestinal concentration of both P-alb and P-IgG remaining 4 h after feeding were high in the group fed colostrum, while only trace amounts were left in the group fed inhibition-free colostrum.

Effect of Feeding Porcine Serum

The rats in the group given porcine serum (TIC 3.7 IU/ml) had an average serum concentration of $28 \mu\text{g/ml}$ for P-alb and $274 \mu\text{g/ml}$ for P-IgG 4 h after feeding (table IIA). The P-alb concentration was doubled, $54 \mu\text{g/ml}$, in the group fed porcine serum with an experimentally elevated TIC value (11.2 IU/ml), while the concentration of P-IgG, $282 \mu\text{g/ml}$, was roughly equal to the value of the other group. No immunoreactive fragments of either P-alb or P-IgG were found in the serum samples investigated. The amount of P-alb and P-IgG remaining in the intestines was considerably higher in the group fed porcine serum with elevated TIC. In this group intestinal TIC was found, while proteolytic activity was observed in the group fed untreated porcine serum.

Effect of Feeding Porcine Serum after Ligation of the Pancreatic Duct

The rats with ligated pancreatic ducts had considerably higher serum values of P-alb, $50 \mu\text{g/ml}$, in comparison to the rats in the sham-operated group, $24 \mu\text{g/ml}$ (table IIB). However, the P-IgG serum concentrations were almost equal in both groups, about $200 \mu\text{g/ml}$. No immunoreactive fragments of

Table II. The mean concentration ($\mu\text{g/ml} \pm \text{SD}$) of porcine albumin (P-alb), and IgG (P-IgG) in the serum, and the proximal and distal portions of the small intestine of 14-day-old rats 4 h after oral feeding

	Num- ber of rats	Serum		Proximal small intestine		
		P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	TIC IU/ml
A Porcine serum	18	25 ± 5	274 ± 45	9 ± 8	t	pa
Porcine serum + SBTI	18	$54 \pm 14^{***}$	$282 \pm 51^{\text{NS}}$	$2,564 \pm 735$	$1,188 \pm 355$	0.1 ± 0.1
B Pancreatic duct ligation	10	$50 \pm 23^*$	$180 \pm 71^{\text{NS}}$	$6,020 \pm 2,896$	$4,965 \pm 2,050$	0.7 ± 0.5
Sham operation	5	24 ± 9	216 ± 70	14 ± 11	t	pa

The trypsin-inhibiting capacity (TIC) values in IU/ml (mean $\pm$ SD) of the intestinal contents are also given.

A The young rats were fed either with porcine serum of low TIC (3.7 IU/ml) or with porcine serum of high TIC (11.2 IU/ml) obtained after addition of soybean trypsin inhibitor (SBTI).

B The rats were subjected either to pancreatic duct ligation or to a sham operation prior to feeding with porcine serum.

Table III. The mean concentration ($\mu\text{g/ml} \pm \text{SD}$) of porcine serum albumin (P-alb) and IgG (P-IgG) in the serum and the proximal and distal portions of the small intestine of 14-day-old rats 4 h after oral feeding with various concentrations of porcine serum

Porcine serum concen- tration %	Num- ber of rats	Serum		Proximal small intestine			Distal small intestine		
		P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	TIC IU/ml	P-alb $\mu\text{g/ml}$	P-IgG $\mu\text{g/ml}$	TIC IU/ml
12.5	1	0	125	t	0	pa	11	0	pa
	2	0	147	t	0	pa	9	0	pa
25	3	0	163	t	0	pa	13	0	pa
	4	0	163	t	0	pa	9	0	pa
50	5	7	203	t	0	pa	2	0	pa
	6	7	203	t	0	pa	5	0	pa
100	7	19	317	12	t	pa	26	t	pa
	8	20	317	6	t	pa	19	t	pa
200	9	56	317	334	t	pa	273	t	pa
	10	62	317	306	t	pa	227	t	pa

The trypsin-inhibiting capacity (TIC) of intestinal contents is also given.

t = Trace amounts; pa = proteolytic activity.

Distal small intestine		
P-alb µg/ml	P-IgG µg/ml	TIC IU/ml
59 ± 41	t	pa
4,478 ± 1,278	1,463 ± 340	0.6 ± 0.1
3,550 ± 1,170	2,905 ± 858	0.3 ± 0.1
24 ± 9	t	pa

t = Trace amounts; pa = proteolytic activity.

Significance in comparison with the rats fed porcine serum in A and the sham-operated rats in B: ***p < 0.001; *p < 0.05; NS (not significant) = p > 0.05 (Student's t test).

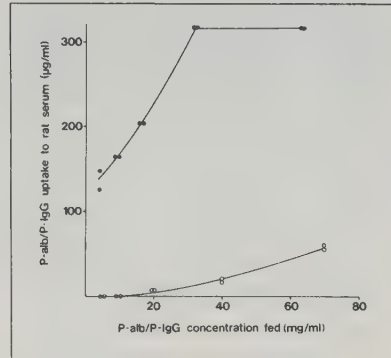


Fig. 1. The uptake of porcine albumin (P-alb, ○) and porcine IgG (P-IgG, ●) in µg/ml into the blood serum of 14-day-old rats 4 h after oral feeding of 0.05 ml porcine serum/g body weight in concentrations of 12.5, 25, 50, 100 and 200%. Each dot represents 1 rat.

either P-alb or P-IgG were found in the serum samples investigated. In the group submitted to pancreatic duct ligation, the intestinal concentrations of both proteins and the TIC value were elevated.

Effect of Feeding Varied Concentrations of Porcine Serum

The results of this experimental series show that the concentration of P-alb found in rat serum 4 h after feeding was proportional, when detectable within the limits of the method, to the dose of P-alb fed (table III). P-IgG, however, was absorbed much more effectively than P-alb and reached a plateau upon feeding high concentrations of porcine serum (fig. 1). No immunoreactive fragments of either P-alb and P-IgG were found in the

serum samples investigated. The small intestine of the rats contained small amounts P-alb and P-IgG, which also increased with the dose given. Proteolytic activity was found in all samples of intestinal contents.

Discussion

From the experiments described in this paper, it is evident that P-alb and P-IgG, the proteins studied, are taken up into the blood stream from food via the gut of 14-day-old rats. These results agree with those of previous investigations on the ability of the suckling rat to absorb proteins of both homologous and heterologous origin through the cells of the small intestinal epithelium [2].

Table IV. The total calculated serum uptake of porcine albumin (P-alb) and IgG (P-IgG) of the young rats in the feeding experiments calculated as percentage of the total dose of the proteins fed, according to the formula: $\frac{\text{serum concentration} \times \text{PV} \times 100}{\text{total dose fed}}$

	Porcine colostrum	Porcine colostrum + trypsin	Porcine serum	Porcine serum + SBTI	Pancreatic duct ligation	Sham- operation
P-alb	0.14	0.06	0.08	0.16	0.15	0.06
P-IgG	0.62	0.60	0.77	0.79	0.50	0.56

The plasma volume (PV) was calculated using 5.53% of the body weight [20].

SBTI = Soybean trypsin inhibitor.

For these feeding experiments, we initially decided to use porcine colostrum, since this can serve both as a source of heterologous proteins and as a source of protease inhibitor. Sow colostrum, in contrast to rat colostrum/milk [3] contains high amounts of a specific trypsin-chymotrypsin inhibitor, the function of which appears to be to protect ingested proteins from gastrointestinal proteolysis, thereby enhancing the nonselective protein absorption in the neonatal piglet [4, 17, 28]. However, after feeding sow colostrum to young rats, some apparent negative effects were observed, such as the heavy clotting of proteins in the stomach and distended gaseous-filled intestines. Therefore the source of proteins was changed to porcine serum. It is true that porcine serum also contains protease inhibitors [27], but some of these would probably be inactivated before reaching the small intestine due to their sensitivity to the relatively low pH of the stomach [9, 15].

It must be emphasized that all feeding experiments, both those performed in the presence of protease inhibitors and those performed in the absence of pancreatic proteases, showed that the proteolytic activity in

the gastrointestinal tract is an important mechanism for regulating the amount of undegraded protein being absorbed into the serum of the suckling rat. That it is the proteolytic activity per se which regulates this protein absorption and not the added protease inhibitors, is convincingly shown by the results of the experiments where the pancreatic duct was ligated.

The total uptake of undegraded P-IgG and P-alb to the serum of the rats was calculated as a percentage of the dose given (table IV), and has to be considered as a minimum value, since some protein escapes from the blood circulation into the extravascular spaces [20]. The uptake of IgG into the serum is about 10 times more efficient than that of albumin, when there is normal proteolytic activity. When the proteolytic activity is diminished, resulting in an increased albumin absorption and an unaffected IgG absorption, the efficiency of the IgG absorption decreases to 5 times that of albumin.

The rather low values for the total uptake of the proteins studied that we obtained in this investigation, as compared with those of other investigators, depends mainly on two

factors. Our experiments were performed under physiological conditions using oral feeding of unanesthetized rats, while most other investigators have injected the proteins into ligated loops of the small intestine, thereby missing the effects of, for example, gastric processing, pancreatic juice influx and intestinal passage. Secondly, we used immunological methods for analyzing the uptake of proteins, thereby selecting for the immunoreactive fraction of the protein being absorbed. Absorption into the serum and especially into the extravascular spaces of digested proteins, including non-immunoreactive fragments, has been shown to occur [12, 19]. In our study, no immunoreactive fragments were found using immunoelectrophoretic methods, except for trace amounts observed in a few rats fed with inhibition-free colostrum. Thus, the great majority of the protein absorbed appeared intact in the blood serum within 4 h after feeding.

The fact that P-IgG was more efficiently transmitted than P-alb to the blood of the young rats focuses interest upon the differences in the mechanisms of uptake of these proteins in the small intestine. Our results show that the uptake of P-IgG is dose dependent up to a concentration of 30 mg/ml, but above this level the absorption reached a maximum since a constant serum level of P-IgG was observed. It is also noteworthy that when oral doses of physiological amounts of IgG are given, similar to the amounts present in rat milk, the specific IgG-uptake appears to be saturated and will not work more efficiently during experimentally lowered proteolytic activity. These results agree with the widely accepted receptor hypothesis which claims that IgG is absorbed via a specific route. IgG combines with selective receptors for the Fc region, thereby escaping proteoly-

sis when endocytosed [2, 22, 25]. These findings also agree well with those of a recent study by Jones [13]. However, when feeding low concentrations of IgG, the uptake appears to be dependent on the gastrointestinal proteolysis.

In comparison, the transmission of albumin from the food into the blood serum increased when the gastrointestinal proteolytic activity was reduced. In addition, the albumin concentration in serum increased proportionally with the dose fed, within the dose range studied. These results can be explained by the increased amounts of albumin available for uptake in the small intestine. It is probable that albumin is absorbed via another route than IgG, perhaps via non-selective endocytosis, as suggested by Wild [29]. This type of protein uptake appears to continue even in adult life [16, 26, 30], but the rate might decline with growth of the rat [11, 14]. The ability to absorb IgG via specific receptors disappears at an age of about 21 days in the rat [2, 22], and from this age the absorption of macromolecules into the serum is supposed to be maintained via basal nonselective endocytosis.

When the rats were fed colostrum or serum with low inhibiting capacity, intestinal proteolytic activity could be detected and only low amounts of the fed proteins remained in the intestines. However, when fed high levels of protease inhibitors or after ligation of the pancreatic duct, no intestinal proteolytic activity could be detected and large amounts of protein remained in the intestine 4 h after feeding.

The concentrations of both P-alb and P-IgG were higher in the proximal than in the distal parts of the small intestine in the experiments where colostrum was given and where the rats were subjected to laparotomy before

feeding. The reverse was true in the experiments where porcine serum plus soybean trypsin inhibitor was given. These results could be explained by a lowered gastrointestinal passage rate due to the negative effects of feeding colostrum, as discussed earlier, or due to the surgical trauma after laparotomy.

The site of proteolysis in the young rat could be intraluminal, by the pancreatic proteases, and/or intracellular, by the lysosomal proteases. Intra-gastric proteolysis is probably not important since the pepsinogen content in the stomach is low until weaning [1, 7]. Porcine colostrum inhibitor has the ability to inhibit both pancreatic proteases such as trypsin and chymotrypsin [17] and intracellular proteases such as granulocyte proteases [24], while soybean trypsin inhibitor only appears to inhibit the proteases in the lumen [13]. In this study, the main effect of the protease inhibitors appears to be on the intraluminal proteolysis since an identical effect on the protein transmission was achieved both by the exclusion of the pancreatic proteases using pancreatic duct ligation and by the use of protease inhibitors.

A physiological role of the nonselective macromolecular transmission, as represented by the transfer of albumin, may be to serve as a mechanism for detection of potential antigens in the gut contents, and appears to be involved in the process of induction of tolerance or intolerance to food proteins [8, 21]. The amount of antigen that provokes the immune apparatus of the organism has been shown to be of importance for the induction of either tolerance or sensitization [10]. The results of the present investigation show that the gastrointestinal proteolytic activity participates in the regulation of the amount of protein which reaches the organism via the gut.

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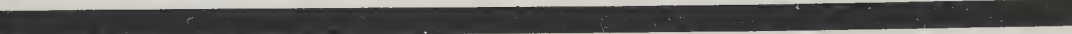
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II



Intestinal Macromolecular Transmission in the Young Rat;
Influence of Protease Inhibitors during Development.

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Running title: Regulation of intestinal macromolecular trans-
mission in the young rat.

Abstract

Intestinal macromolecular transmission in young rats aged 10, 14, 18, 22 and 30 days was measured as the blood serum levels of markers 6 h after orally feeding a solution containing bovine IgG (BIGG), bovine serum albumin (BSA) and fluorescein-isothiocyanate labelled dextran 70,000 (FITC-D), either alone (controls) or with soybean trypsin inhibitor (SBTI) or swine-colostrum trypsin inhibitor (SCTI).

In the 10 and 14 days old rats, transmission of all three macromolecular markers was high, with a preference for IgG. Transmission was greatly reduced by the age of 18 days and totally arrested for the protein markers at 22 days, with a low transmission of FITC-D remaining at 30 days of age. Addition of either of the two protease inhibitors significantly elevated the transmission of the protease-dependent markers in the rats aged 10, 14 and 18 days, while the transmission of FITC-D was unaffected. From 14 days of age the rats have a functioning intestinal proteolysis since only small amounts of marker proteins were left in the gut lumen 6 h after feeding, and since the addition of protease inhibitors resulted in increased amounts of undegraded proteins intraluminally.

The results indicate that the increase of intraluminal proteolytic activity during development and the presence of protease inhibitors in the food, is of importance for the intestinal transmission of undegraded proteins in the young rat. The Fc-receptor for IgG in the enterocyte is not sufficient to maintain an optimal transmission of IgG since the intraluminal proteolytic activity also appears to be of importance.

Introduction

Macromolecules are readily transmitted across the gut epithelium in fetuses and neonates of several mammalian species, but this ability is greatly reduced (intestinal closure) during the ontogeny. In the adult only minute amounts of macromolecules are transmitted (Brambell, 1970; Walker, 1981; Dahl et al., 1984; Telemo et al., 1986). A result of elevated transmission is that the offspring is provided with maternal immunoglobulins and other macromolecules from colostrum or milk. During passage along the gastrointestinal tract and through the gut epithelium, the colostrum/milk proteins must be protected from proteolytic degradation to retain their physiological functions.

Two different types of postnatal intestinal transmission of macromolecules have been described. In ungulates, a massive endocytosis and transmission of virtually all intraluminal macromolecules takes place (nonselective transmission) during the first day of life (Hardy, 1969; Brambell, 1970; Weström et al., 1984). During transmission the macromolecules are protected from proteolytic degradation by protease inhibitors present in colostrum (Laskowski et al., 1957; Carlsson et al., 1980; Weström et al., 1982). In the newborn pig, this results in an effective transmission of immunoglobulins and other macromolecules from colostrum to the blood (Jensen and Pedersen, 1979; Weström et al., 1985).

In neonatal rodents, immunoglobulins (IgG) are endocytosed and selectively transmitted to the blood after binding to specific Fc-receptors on the enterocytes in the proximal small intestine (Rodewald, 1973; Morris, 1976; Mackenzie, 1984). Other

macromoleculese are transmitted simultaneously in small amounts (Jones, 1974; Morris et al., 1981; Telemo et al., 1982). In the rat this macromolecular transmission lasts three weeks. Rat milk has a relatively low protease inhibiting capacity (Carlsson et al., 1975) compared with the ungulates, and IgG could escape degradation by binding to the receptors.

The aim of the present investigation was to elucidate macromolecular intestinal transmission in young developing rats by feeding three kinds of macromolecular markers with different properties: bovine IgG (BIgG), which is degradable in the intestines and has a receptor dependent transmission; bovine serum albumin (BSA), degradable and receptor-independent; FITC-Dextran 70,000 (FITC-D), nondegradable and receptor independent. The markers were fed together, with or without protease inhibitors to evaluate the influence of the intestinal proteolytic system on macromolecular transmission.

Materials and methods:

Ten litters of Sprague-Dawley strain rats 10, 14, 18, 22 and 30 days old (two litters of each age) bred under standard conditions were used. The young were separated from their dams, placed in heated cages and fasted two hours followed by gavage feeding of 0.05 ml of marker solution/g b.w. via a stomach tube. The macromolecular marker solution contained BSA (fraction V; Sigma, St.Louis, Mo, USA), bovine gammaglobulins (Cohn fraction II, Sigma) and FITC-labelled Dextran with a mean MW of 70,000 daltons (FITC-D, Sigma), in a concentration of 30 mg/ml of each in saline. Each litter was divided into three groups.

One group (controls) was fed only the marker solution while the other two groups were fed the marker solution with soybean trypsin inhibitor, (SBTI, Sigma), 2 mg/ml or purified swine colostrum trypsin inhibitor, SCTI, (Laskowski et al., 1957), 6 mg/ml. At the given concentration each ml of inhibitor containing marker solution, inhibited 3.5 mg of porcine trypsin (Novo Ind., Copenhagen, Denmark). After feeding, the rats were kept separated from their dams for a further six hours and then killed by an overdose of Mebumal.

Blood obtained by heart puncture was allowed to clot at room temperature. After centrifugation at 3000 x g for 10 minutes, the serum was removed and saved. Urine was taken by puncturing of the urinary bladder. The stomach, the small intestine (divided into one proximal and one distal portion of equal length) and the liver were removed from the carcass immediately after death. The contents of the stomach and each portion of the intestine were flushed out with 1 ml ice-cold saline. The liver was homogenized in icecold saline (1:1 w/v) by ten strokes in a teflon glass homogenizer, centrifuged at 3000 x g at +4°C and the supernatant was saved.

All samples were stored at -20°C until analysis.

Analysis

Quantitation of BSA was performed by electroimmunoassay (Laurell, 1966) using purified BSA (A 7638; Sigma) as a standard and a specific antiserum obtained from Miles Laboratories (Freehold, NJ, USA). The BIGG concentration was estimated using single radial immunodiffusion (Fahey and McKelvey, 1965) using

purified BIGG (G-3500; Sigma) as standard and a specific anti-serum from Miles. Serum was analyzed for the FITC-D concentration by fluorescence spectrometry as described elsewhere (Weström et al., 1984).

Trypsin inhibiting capacity (TIC) of the stomach and gut samples was determined spectrophotometrically using benzoyl-DL-arginine-p-nitroanilide (Sigma) as a substrate for trypsin (Carlsson and Karlsson, 1972). One inhibiting unit (IU) corresponds to an inhibition of 0.4 mg porcine trypsin. Negative TIC values were taken as evidence for proteolytic activity.

Results

Marker levels in blood serum

As seen in the Table, the serum levels of the protein markers BIGG and BSA were high 6 hours after feeding in the 10-14 days old rats, but were significantly reduced in the 18 days old rats. In the 22 and 30 days old rats no marker proteins were detected, except for small amounts in three individuals. The average serum concentration of BIGG was 15-30 times that of BSA.

Feeding the marker solution with the protease inhibitors SBTI or SCTI, elevated the serum levels of BIGG and BSA in 10, 14 and 18 days old rats as compared with the controls. This was significant ($p < 0.05$) for all groups, except for IgG in 10 days old rats and for BSA in the 18 days old rats in the SBTI group.

FITC-D was found in the serum of all age groups, but the levels were considerably lower in the 22 and 30 days old rats.

The transmission of the protease independent marker FITC-D was not influenced by the addition of protease inhibitors.

Markers remaining in gastrointestinal contents.

In the 10 days old rats, large amounts of both B1gG and BSA were found in the stomach contents of all three feeding groups. The proteins were also found in intestinal contents, with the highest levels in the proximal part. The SBTI and SCTI groups had higher concentrations of the proteins in both portions of the small intestine than the controls. In the 14 days old rats, the amounts of B1gG and BSA remaining in the stomach and intestinal contents were considerably lower, but the SBTI and SCTI fed rats showed higher concentrations in the digestive contents than the controls. In the 18-30 days old rats, only traces of BSA and B1gG were found occasionally in both the controls and the SBTI and SCTI groups. No apparent differences were found between the SBTI and the SCTI groups at any age.

Markers in urine and liver samples.

B1gG was found in urine from 10, 14 and 18 days old rats, but in amounts below 5 µg/ml. Occasionally, BSA was also found in amounts below 4 µg/ml.

In the liver homogenates, no B1gG or BSA was found in any of the feeding groups or developmental stages studied.

Effect of protease inhibitors on the intraluminal proteolytic activity.

With increasing age all feeding groups showed increasing trypsin activity in the gut contents: from 0.0 to 0.1 EU/ml in the stomach contents and from 0.0 to 0.25 EU/ml in the proximal

and distal small intestinal contents between 10 to 30 days of age. The trypsin activity did not differ significantly between the feeding groups, except for 18 days old rats fed SBTI which showed a decreased activity (0.06 ± 0.02 EU/ml) as compared to the controls (0.1 ± 0.02 EU/ml).

Discussion

The intestinal transmission of each of the three markers evaluated as the serum levels 6 hs after feeding, showed principally the same pattern during postnatal development. The serum levels of BIgG (approx. 25-fold those of BSA) were taken as evidence for selective intestinal transmission of IgG in the young rat. It has also previously been shown that heterologous IgG's are transported via receptor-mediated transmission in the enterocytes although not as readily as homologous IgG (Morris et al., 1981). Our results show that the IgG transmission ceases (intestinal closure), at the age of three weeks and that the diminished IgG transmission coincides with a diminished nonselective transmission as shown by the receptor-independent markers BSA and FITC-D. While the nondegradable marker FITC-D probably indicates the extent of the endocytotic activity and transport through the enterocytes per se, BSA also mirrors the influence of the gastrointestinal proteolytic activity. Despite the fact that equal amounts of FITC-D and BSA were fed and that FITC-D is resistant to proteolytic degradation a considerably lower serum concentration of FITC-D was seen. This discrepancy could reflect qualitative differences in the transmission of the two markers. It should be noted, however, that

the elimination of FITC-D from the circulation after being injected i.v. is much more rapid than that of BSA. The estimated half life of FITC-D in the 14 days old rat is about 3 h, and for BSA about 12 h (unpublished observations). It was possible to elevate the transmission of the protein markers BIGG and BSA, but not FITC-D, in the "pre-closure" rat by feeding protease inhibitors with the marker. This result does not agree with the results of Jones (1980) who could not elevate the transmission of human IgG in a rat small intestinal loop by adding SBTI. Judging from the present data it appears as if the decline in the IgG transmission from 14 to 18 d of age depends on increased proteolytic activity in the gut lumen. At 22 days and thereafter, the transmission of all markers was remarkably reduced, independent of the presence of proteinase inhibitors, indicating a decline in the transmission capacity of the enterocytes.

It is obvious that the young rat, at least from 14 days of age, has a functioning gastrointestinal proteolytic system, as judged from the small amounts of undegraded proteins remaining in the gut content 6 hours after feeding (Table). This agrees very well with studies on the enzyme development in the suckling rat (Henning 1981; Davies and Messer, 1984). The efficiency of this proteolytic system appears to be dependent on the protease inhibitors present in the test solutions fed. No difference was seen between the two inhibitors used, though SBTI mainly functions as a trypsin inhibitor while SCTI functions both as a trypsin and chymotrypsin inhibitor (Weström et al, 1985). In addition, SCTI inhibits some intracellular proteases, such as granulocyte proteases (Stancikova et al, 1980), while SBTI appears to lack this ability (Jones, 1980).

In many species an important ingredient of colostrum and milk is protease inhibitors. Sow's colostrum for example contains the specific protease inhibitor, SCTI, in high amounts (7-8 IU/ml), which has been shown to enhance the transmission of proteins from colostrum to blood serum of the piglet (Jensen and Pedersen, 1979, Weström et al, 1985). Rat colostrum, however, exhibits low inhibiting capacity the first 12 h of lactation but the level increases in the milk to approximately 1 IU/ml, which is maintained throughout most of the lactating period (Carlsson et al, 1975). Our results imply that the presence of protease inhibitors in the gut of the pre-closure rat increases the availability of proteins in the small intestine and thereby makes the transmission of these molecules more effective. However, it is not clear whether the natural protease inhibition of rat milk, which is lower than the inhibition in the experimentally fed test solutions added with inhibitors (7.5 IU/ml), is sufficient to exert this effect in the suckling rat.

The trypsin inhibitors used in this study and possibly also those naturally occurring in rat milk constitute extracellular factors regulating the proteinase activity in the gut lumen and thus the amount of proteins available for endocytosis by the enterocytes. Our results show that the Fc-receptor on the enterocytes is not sufficient per se to give an optimal transmission of IgG and that the protein transmission in general is affected by the presence of protease inhibitors in the food of the neonate.

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Heading to the table

The mean serum concentration ($\mu\text{g/ml} \pm \text{SD}$) of the markers B1gG, BSA, FITC-D 70,000 and the median concentrations (with range within brackets below) of B1gG and BSA remaining in the stomach, proximal and distal parts of the small intestine of 10-30 days old rats, 6 h after oral feeding. In the groups SBTI and SCTI the marker solution included SBTI 2 mg/ml or SCTI 6 mg/ml.

Level of significance in comparison to control rats:

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ (Student's t-test)

+ = trace amounts

n.d. = not detectable; The detection limit for B1gG was 4 $\mu\text{g/ml}$, for BSA 1 $\mu\text{g/ml}$ and for FITC-D 0.1 $\mu\text{g/ml}$.

1 = amount found in one rat.

Legend to the figure

Mean serum concentration ($\mu\text{g/ml}$) of B1gG, BSA, and FITC-D in 10, 14, 18, 22 and 30 days old rats, 6 hs after feeding.

 = controls

 = SBTI

 = SCTI

small intestine
proximal

stomach

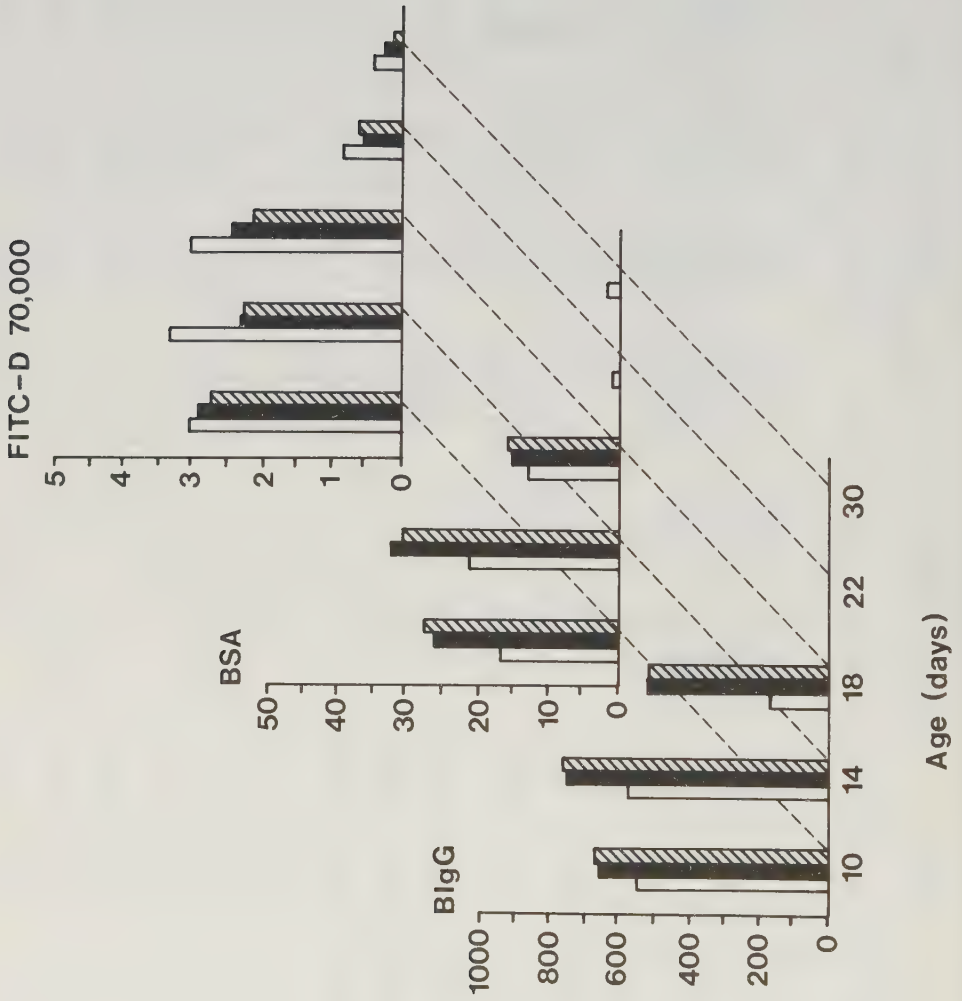
serum

Age Group

days

distal

days		BIgG	BSA	FIITC-D	BIgG	BSA	BIgG	BSA	BIgG	BSA
10	contr (6)	551±89	17 ±8	3.0±0.3	1200 (930-1450)	1200 (960-1530)	190 (+250)	74 (+155)	170 (48-430)	22 (+35)
	SBTI (6)	658±152	26±5*	2.9±0.2	1520 (1340-1700)	1090 (610-1340)	460 (290-850)	430 (310-490)	320 (130-650)	290 (+440)
	SCTI (6)	664±60*	27 ±11*	2.7 ±0.6	1620 (1490-1730)	860 (530-990)	390 (100-630)	280 (120-310)	300 (90-610)	190 (90-270)
14	contr (6)	577±127	21±7	3.3±0.8	10 (+16)	7 (4-9)	8 (+12)	5 (1-13)	9 (+14)	2 (+5)
	SBTI (6)	748±98*	32 ±2**	2.3±0.5	95 (30-150)	65 (44-86)	23 (+61)	17 (6-23)	20 (+48)	21 (5-41)
	SCTI (6)	752±139*	30±5*	2.3±0.4	82 (24-210)	74 (41-98)	53 (12-84)	16 (5-19)	17 (+34)	12 (2-17)
18	contr (7)	170±64	13±2	3.0±1.5	16 (+26)	+	11 (+13)	+	+	n.d.
	SBTI (6)	518±112***	15±3	2.4±0.6	19 (+32)	+	6 (+9)	+	+	+
	SCTI (6)	520±181***	16±3*	2.1±0.5	+	+	7 (+12)	n.d.	n.d.	n.d.
22	contr (6)	n.d.	4 ¹	0.8±0.2	16 (+23)	+	+	n.d.	+	n.d.
	SBTI (5)	n.d.	n.d.	0.5±0.3	23 (+42)	+	14 (+23)	n.d.	n.d.	n.d.
	SCTI (5)	n.d.	n.d.	0.6±0.3	+	+	n.d.	n.d.	n.d.	n.d.
30	contr (8)	n.d.	12 ¹	0.4±0.2	+	n.d.	n.d.	n.d.	n.d.	n.d.
	SBTI (6)	4 ¹	n.d.	0.2±0.2	n.d.	n.d.	+	n.d.	n.d.	n.d.
	SCTI (6)	n.d.	n.d.	0.1±0.1	+	+	n.d.	n.d.	n.d.	n.d.





THE PASSAGE OF ORALLY FED PROTEINS FROM MOTHER TO FOETUS IN THE RAT

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Abstract—1. Pregnant rats were orally fed with a mixture of bovine IgG, bovine albumin, ovalbumin and β -lactoglobulin. Using immunoprecipitation methods, these proteins were detected in the maternal blood serum, urine and uterine fluids, and also in the foetal blood serum and the amniotic fluid.

2. The results imply that a variety of dietary proteins, still immunoprecipitable, are able to cross the materno-foetal barriers to be detected in the foetal circulation, where they may influence the maturation of the immune system of the foetus.

INTRODUCTION

Transmission of IgG from the bloodcirculation of the mother to the foetus occurs in many species, including the rat (Brambell, 1970). This is a route by which the foetus acquires passive immunity providing an immediate protection to common antigens, e.g. infectious agents, in the extrauterine environment before its own immune system is developed. In the rat, even orally fed ¹²⁵I-labelled IgG and its breakdown products are transferred from the gut of the mother to her foetuses (Hemmings and Hemmings, 1979).

This work is an attempt to investigate whether proteins other than IgG, when orally fed to the mother, can cross the tissue barriers between the mother's gut and the foetus and reach the circulation of the foetus. The proteins chosen for feeding were common dietary proteins having different properties, i.e. molecular weight, and known to give rise to food allergy in man (Bleumink and Young, 1968). To detect and quantify the transmission of these proteins, immunoprecipitation methods were used. These methods may give a better picture of the uptake of proteins than the methods using radio-labelled tracers commonly in use since the latter methods can result in an overestimation of the protein uptake (Udall *et al.*, 1981).

MATERIALS AND METHODS

Nineteen female rats of the Sprague-Dawley strain (Anticimex, Stockholm, Sweden) aged about 3 months and bred at our laboratories under standard conditions, were employed in the study. Fifteen of them were pregnant between days 10 and 22, and four remained virgins. The gestational age was estimated by measuring the crown-rump length of the foetuses (Angulo, 1932).

The rats had free access to food and water until the experimental feeding, which was performed at 9 a.m. The animals were fed bovine serum, which contained 55 mg/ml of bovine IgG (BlgG) and 66 mg/ml of bovine serum albumin (BSA), mixed with the proteins chicken ovalbumin (OvA) (Sigma, St Louis, MO, USA) and cow milk β -lactoglobulin (β -LG) (Sigma) to a concentration of 30 mg/ml for both of these proteins. Each rat received 3.33 ml of this protein solution per 100 g body weight. The rats were fed under light ether anaesthesia by means of a stomach tube.

Four hours after the protein feeding, the rats were anaesthetized (Mebumal, 40 mg/kg body weight) and laparotomy was performed. Blood serum was obtained from the adults by heart puncture and from the foetuses (pooled within the litter) by bleeding after decapitation. The contents in the small intestine of the adult were obtained by flushing the proximal 20 cm with 2 ml of ice-cold 0.9% NaCl, and the supernatant was collected after centrifugation at 3000 g for 15 min. Adult livers were homogenized in 1 ml ice-cold 0.9% NaCl per g tissue in a Teflon-glass homogenizer. The homogenates were centrifuged at 3000 g for 15 min and the supernatant was saved for analysis. Samples of uterine and amniotic fluids, and urine were taken when possible.

All samples were stored frozen (-20°C) until analysis.

Detection and quantitation of the proteins were performed by electroimmunoassay (Laurell, 1966) for BSA, OvA and β -LG, and by single radial immunodiffusion (Fahey and McKelvey, 1965) for BlgG. Specific antisera for BlgG and BSA were obtained from Miles Laboratories, Freehold, USA and antisera for OvA and β -LG were raised in rabbits as described elsewhere (Jakobsson *et al.*, 1982).

RESULTS AND DISCUSSION

The results of this investigation show that food proteins, still immunoprecipitable, have the ability to cross the intestinal membranes into the blood of the rat, both pregnant and non-pregnant, and even to reach the circulation of the foetus.

In the gastrointestinal tract of the adult rats, the degradation and elimination of the fed proteins must be pronounced, since only small amounts of immunoprecipitable proteins, representing less than 1% of the dose given, could be detected within four hours after feeding (Table 1). Somewhat higher amounts of ovalbumin than the other proteins were found in most intestinal samples, whereas BSA and β -LG occurred only in a few intestinal samples in low amounts. The IgG molecules appeared fragmented in the intestines, as evaluated from the multiple rings found in the single radial immunodiffusion. These results indicated that there were differences in the availability for absorption of the various protein molecules in the gut. One reason for this could be differences in resistance of the different proteins to proteolytic degradation.

Table 1. Mean concentration $\pm$ SD in $\mu\text{g/ml}$ (calculated for the samples with detectable amounts of protein) for the proteins bovine IgG (B1gG), bovine serum albumin (BSA), ovalbumin (OvA) and β -lactoglobulin (β -LG) in intestinal samples, blood serum, urine, uterine and amniotic fluids from adult and foetal rats

	Intestine	Bloodserum	Urine	Uterine fluid	Amniotic fluid
Adults, non-pregnant					
B1gG	DP (4/4)	+	+		
BSA	5.0 (1/4)	7.1 $\pm$ 3.9 (4/4)	12.0 $\pm$ 8.3 (3/4)		
OvA	38.2 $\pm$ 16.6 (4/4)	11.5 $\pm$ 11.7 (4/4)	— (0/4)		
β -LG	— (0/4)	12.7 $\pm$ 6.7 (2/4)	— (0/4)		
Adults, pregnant					
B1gG	DP (14/15)	+	+	+	
BSA	12.1 $\pm$ 8.6 (4/15)	9.2 $\pm$ 4.1 (12/15)	4.5 $\pm$ 3.5 (9/15)	17.2 $\pm$ 1.1 (2/6)	
OvA	34.1 $\pm$ 19.6 (11/15)	9.6 $\pm$ 5.4 (13/15)	— (0/15)	11.2 $\pm$ 3.8 (3/6)	
β -LG	12.0 $\pm$ 3.5 (2/15)	13.4 $\pm$ 7.5 (10/15)	11.3 $\pm$ 6.5 (4/15)	— (0/6)	
Foetuses					
B1gG		+			+
BSA		9.5 $\pm$ 9.2 (6/15)			30.0 (1/7)
OvA		5.7 $\pm$ 4.9 (6/15)			13.7 $\pm$ 10.2 (2/7)
β -LG		— (0/15)			— (0/7)

Figures in brackets indicate the ratio between samples with detectable amounts of the protein and the total number of samples analysed.

DP, degradation products.

+, trace amounts ($< 4 \mu\text{g/ml}$).

—, no detectable amounts.

In the blood sera of both pregnant and non-pregnant rats, the proteins fed could be detected and quantified, showing the ability of food proteins to cross the intestinal membranes (Table 1). The results showed no apparent differences in transmission between pregnant and non-pregnant rats. The amounts of the fed proteins in the sera varied considerably, both between different proteins and between individual rats (0–29 $\mu\text{g/ml}$). However, in one and the same rat the various proteins were absorbed in either relatively high or in relatively low amounts. These differences in protein transmission could be explained by differences in the intestinal proteolytic activity and/or endocytotic efficiency of the gut cells, or differences in elimination rates. In addition, food intake before the start of the experiment could affect the time of passage through the intestines, and thus the availability for absorption.

The differences observed in the uptake of the various proteins did not appear to be due to differences in the molecular weights of the respective proteins (B1gG MW: 150,000, BSA MW: 67,000, OvA MW: 43,000, β -LG MW: 18,500).

In a few liver samples, small amounts of immunoprecipitable proteins appeared. BSA was found in one liver sample at a concentration of 9.5 $\mu\text{g/ml}$, and OvA in another at a concentration of 15 $\mu\text{g/ml}$. These observations, all obtained on liver supernatants, did not agree with the high values reported by Hemmings

and Hemmings (1979), who observed that the liver homogenates had the greatest uptake of ^{125}I -IgG fed to rats. The method of detection used by these investigators can also detect radio-labelled breakdown products of the fed protein whereas in our study only intact protein and relatively large, still immunoprecipitable protein products have been measured.

In the foetal blood sera, detectable amounts of B1gG, BSA and OvA were found in almost half of the samples (Tables 1 and 2). There was no major difference between the transmitted amounts of B1gG and the other proteins (BSA and OvA). This is remarkable since a selectivity for IgG via receptor-mediated uptake would have been expected (Kraehenbuhl *et al.*, 1979). Another interesting observation is that the concentration of BSA and OvA in foetal serum sometimes exceeded the concentration found in maternal serum (Table 2). These observations indicated that there was an accumulation of the absorbed proteins in the rat foetus. In addition, there was an increased concentration of the proteins in foetal sera from late gestation.

As found in this study, the rat foetus can develop in the presence of immunogenic dietary proteins derived from the mother. It is interesting to speculate about the roles of food proteins in the maturation of the immune system of mammals. One effect may be an induction of tolerance *in utero* to common food stuffs, which could

Table 2. Concentration in $\mu\text{g/ml}$ of bovine serum albumin (BSA) and ovalbumin (OvA) in paired maternal and foetal blood sera (pool of the litter)

Rat No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Day of gestation	10	10	10	11	15	16	16	17	18	19	21	22	22	22	22
BSA															
Maternal bloodsera	12	10	8	8	4	—	6	20	11	8	10	8	—	8	+
Foetal bloodsera	—	—	—	—	—	—	—	2	21	2	21	—	2	9	—
OvA															
Maternal bloodsera	12	10	10	10	1	12	6	15	8	7	8	24	—	5	—
Foetal bloodsera	—	—	—	—	—	—	3	—	3	11	1	3	13	—	—

—, no detectable amounts.

+, trace amounts ($< 2 \mu\text{g/ml}$).

be of advantage to the animal. However, in special cases, particular foreign proteins could induce intolerance, resulting in a sensitization *in utero* (cf. Jarett, 1977).

High levels of BSA and OvA were also observed in some of the amniotic and uterine fluid samples. These samples came from the late gestational stages, indicating a possible transfer route via the uterine and amniotic fluids. After the foetus swallowed the amniotic fluid, the proteins studied may have entered the circulation of the foetus via the gut. In fact, it has been shown for the rat and other mammals, that proteins experimentally deposited in the amniotic fluid enter the foetal circulation via the gut (Leissring and Anderson, 1961; Lev and Orlic, 1973; Orlic and Lev, 1973). However, in our study there was no indication of higher serum levels of BSA and OvA in foetuses surrounded by amniotic and uterine fluids containing high amounts of these proteins. Studies are now in progress to evaluate further the transfer routes of proteins from mother to foetus in the rat.

It has been shown in man that food proteins, including the proteins used in this study, are transmitted to the milk in lactating mothers, resulting in symptoms appearing in their breast-fed infants (Jakobsson and Lindberg, 1983). A similar transfer of proteins as seen in the rat appears to be probable between the pregnant woman and her foetus. For understanding the biological significance of this protein transmission in man the rat may be a useful animal model.

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IV

Transfer of Orally or Intravenously Administered Proteins to the Milk of the Lactating Rat

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Summary: Lactating rats were given a test solution containing various marker proteins via oral or intravenous routes. Using immunoprecipitation methods for the detection of ovalbumin (OvA), bovine serum albumin (BSA), and bovine gammaglobulins (BigG) and radioimmunoassay for the analysis of β -lactoglobulin (β -LG), these proteins could be found in the blood serum and the milk of the rat 6 h after administration. A specific distribution pattern between the serum and the milk was observed for each protein. The results show that orally fed dietary proteins are able to cross

the gut of the lactating rat and can be found in the serum and the milk. It was also observed that oral feeding of the proteins resulted in relatively greater concentrations in the milk than did intravenous administration. This very early presentation to the young of antigenic macromolecules derived from the mother's diet might be of importance for the development of a proper immunological response to common food antigens. **Key Words:** Transfer—Proteins—Lactating rat—Milk.

The observation that undigested proteins can cross the gut and enter the blood circulation of both young and adult mammals is of great interest from both physiological and immunological points of view (1-5). The proteins enter the organism via the intestinal epithelium, probably by means of an endocytotic mechanism in the enterocytes. It has been shown by many authors that fetuses, premature infants, and neonatal individuals transmit greater amounts of undegraded or partially degraded proteins than do adults (6-14). This pattern is often claimed to be due to an undeveloped or immature digestive system and/or more intense endocytotic activity in the young individual.

The amount of proteins transmitted may be sufficient to produce an immunological response in the organism. It has been shown that such antigens as common dietary proteins, even in minute amounts, can evoke immunological reactions leading to the production of IgE antibodies in the adult rat (15,16) and can cause severe anaphylactic reactions in guinea pigs (17) and humans (18). However, orally fed antigens have been shown by other workers to induce systemic

tolerance in various species (19-27). The sensitivity of this system was recently demonstrated by Jacobsson and Lindberg (28) while investigating infant colic in breast-fed infants. They have shown that dietary proteins in the mother's food can cause an allergic reaction in the nursing baby.

As part of our research into the intestinal absorption and transmission of macromolecules and their physiological significance in mammals, we have recently shown that proteins fed orally to the pregnant rat can be detected in the blood circulation, in the amniotic fluid, and in the blood serum of the fetuses (1). The present investigation was undertaken in an attempt to determine whether or not dietary proteins with different properties can be transferred into the milk of the lactating rat, thus exposing the nursing young to proteins from the mother's food.

MATERIALS AND METHODS

Animals

Twenty-three lactating rats (300-375 g) of the Sprague-Dawley strain (Anticimex, Stockholm, Sweden), with 14-day-old litters bred and maintained under standard conditions, were used in this experiment.

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Thirteen dams were fed orally and another 10 dams were injected intravenously with the test protein solutions. The rats fed orally were restricted food for 12 h prior to feeding, but had free access to water.

Protein Administration

The test protein solution consisted of bovine serum (obtained from cows at slaughter) supplemented with chicken ovalbumin (OvA) (Sigma Chemical Co., St. Louis, MO, USA) and β -lactoglobulin (β -LG) from cow milk (Sigma). The concentration of the tested proteins in this mixture was 66 mg/ml bovine serum albumin (BSA), 55 mg/ml bovine IgG (BIgG), and 30 mg/ml each of OvA and β -LG. It should be noted that the rats had not been in contact with these proteins before the experimental feeding.

The orally fed rats received 3.0 ml/100 g body weight of this test solution using a stomach tube while under light ether anesthesia. The intravenously administered rats received 0.33 ml protein solution diluted 1/30 in saline per 100 g body weight. This was injected into the dorsal metatarsal vein during ether anesthesia. After the dams were given the test solution they were returned to their cages and the litters were removed to avoid suckling.

Sampling

Six hours after the administration of the test solution the rats were anesthetized with Mebumal, 30 mg/kg body weight i.p., and given oxytocin (Partocon, Ferring, Sweden) 10 IU/kg i.p. 5–15 min before the milk was collected. This was done using a modified breast pump ("Syster Maja," Einar Egnell AB, Trollhättan, Sweden) that was originally made for human purposes. The time for milking varied from 30 to 45 min, and the average yield was 2 ml per dam. The milk was centrifuged at 20,000 g for 60 min and the fat-free part of the supernatant was saved. After milking, blood samples were taken from the rats by heart puncture and were allowed to clot at room temperature before the serum was harvested. The rats were killed immediately after the blood sampling.

For the orally fed rats samples were also taken from the stomach contents, the proximal and distal small intestines, and the urine. The stomach was washed out with 2 ml ice-cold 0.15 M NaCl and the contents collected. Twenty centimeters of the most proximal and the most distal parts of the small intestine were each flushed with 1 ml ice-cold 0.15 M NaCl. Urine was collected by puncturing the urinary bladder.

All samples were stored at -20°C until analysis.

Analysis

Bovine serum albumin and OvA were quantified by electroimmunoassay (29) using rabbit antisera specific for each protein (Miles, Slough, UK). Purified BSA and OvA (Nos. A 7638 and A 7641, Sigma) dissolved in 0.15 M NaCl were used as standards, and the detection limits for BSA and OvA were 1 $\mu\text{g/ml}$ and 0.2 $\mu\text{g/ml}$, respectively. Possible electrophoretic heterogeneity of BSA in selected milk and serum samples was also checked by crossed-immunoelectrophoresis (30). In addition, immunological identity was controlled by adding standard protein to the samples analyzed in the crossed-immunoelectrophoresis. The β -LG in milk and serum was analyzed by radioimmunoassay (RIA) (31) using a rabbit antiserum specific to β -LG (32) and having a detection limit of 1 ng/ml. This analysis was made possible by the kind courtesy of Dr. Irene Jacobsson, Malmö General Hospital, University of Lund, Sweden. Electroimmunoassay was used for the detection of β -LG in the intestine and urine samples, with a detection limit of 1 $\mu\text{g/ml}$.

BIgG was analyzed by single radial immunodiffusion (33), using a rabbit antiserum specific to BIgG (Miles Laboratories, Freehold, NJ, USA). Purified bovine IgG (No. I 5506, Sigma) dissolved in 0.15 M NaCl was used as a standard, and the detection limit for this method was 4 $\mu\text{g/ml}$.

With the immunoprecipitation and RIA methods described above, there was no observed cross-reactivity in serum and milk samples from normal control rats. When using immunoprecipitation methods for the detection of BSA in serum, there were indications of an overestimation of the absolute concentration, as dissolving the standard proteins in control serum gave somewhat larger precipitates (10–40%) as compared with dissolution in 0.15 M NaCl. It should also be noted that BSA and OvA standards dissolved in rat milk gave weaker and more irregular precipitates, resembling the precipitates seen in the samples, resulting in difficulties in the exact estimation of the absolute concentrations. These kinds of methodological problems were not noted for the detection of BIgG and β -LG. For practical reasons and for obtaining standardized conditions in the analysis, all standard proteins were dissolved in 0.15 M NaCl.

RESULTS

The results show that orally fed proteins can be detected in the serum and the milk of the lactating rat within 6 h after feeding.

Oral Feeding of Test Proteins

The results of the analyses of β -LG, Ova, BSA, and BIgG in serum and milk are presented in Table 1. Using the RIA method, β -LG could be detected in all serum samples with a median concentration of 0.060 μ g/ml and a range of 0.010–0.500 μ g/ml, and in all milk samples with a median concentration of 0.400 μ g/ml (0.090–3.500 μ g/ml). The concentration of β -LG in milk was higher than in serum for all the rats except one (No. 3). Using the less sensitive method, electroimmunoassay, Ova could not be detected in any of the serum samples, but was found in the milk samples from five rats (5/13) at a median concentration of 3.8 μ g/ml (0.6–5.8 μ g/ml). A median concentration of 4 μ g/ml BSA (3–5 μ g/ml) could be detected in the serum of three rats (3/13) and in the milk (1–8 μ g/ml) of five rats (5/13). Using radial immunodiffusion, BIgG could not be detected in any of the serum or milk samples of this group.

The crossed-immunoelectrophoresis analysis of the BSA content of the milk and serum samples in this

group showed that there was no difference in electrophoretic mobility of the main fraction of BSA as compared to the standard protein. However, both the milk and the serum contained a small fraction of BSA with a somewhat reduced electrophoretic mobility.

Analysis of the intestine and urine samples gave negative results for most of the fed rats. Only rats 1 and 2 showed levels of approximately 1 mg/ml for BSA, Ova, and β -LG in the stomach samples, and rats 8 and 12 showed levels of approximately 10 μ g/ml of BSA and Ova in the samples from the distal small intestine. Most rats with undigested proteins in the stomach and/or intestine samples also showed high concentrations of these proteins in serum and milk.

Intravenous Administration of Test Proteins

The serum and milk concentrations of BSA, Ova, BIgG, and β -LG 6 h after intravenous administration are shown in Table 1. The median serum value was 0.182 μ g/ml for β -LG, ranging from 0.108 to 0.600 μ g/ml, and 0.5 μ g/ml for Ova (0.2–0.8 μ g/ml),

TABLE 1. Levels in μ g/ml of β -LG, Ova, BSA, and BIgG in serum and milk of lactating rats

Rat	β -LG		Ova		BSA		B-IgG	
	Serum	Milk	Serum	Milk	Serum	Milk	Serum	Milk
6 h after oral feeding								
1	0.140	0.800	<0.2	5.8	3	8	<4	<4
2	0.130	0.500	<0.2	4.7	<1	3	<4	<4
3	0.500	0.135	<0.2	<0.2	<1	<1	<4	<4
4	0.045	0.090	<0.2	<0.2	<1	<1	<4	<4
5	0.025	0.200	<0.2	<0.2	<1	<1	<4	<4
6	0.123	0.415	<0.2	<0.2	<1	<1	<4	<4
7	0.060	0.200	<0.2	<0.2	<1	<1	<4	<4
8	0.010	3.500	<0.2	3.8	<1	5	<4	<4
9	0.075	0.135	<0.2	0.6	<1	<1	<4	<4
10	0.010	0.400	<0.2	1.0	<1	1	<4	<4
11	0.040	0.100	<0.2	<0.2	5	<1	<4	<4
12	0.100	0.400	<0.2	<0.2	3	<1	<4	<4
13	0.010	0.135	<0.2	<0.2	3	1	<4	<4
Median	0.060	0.400		3.8	4	4		
6 h after intravenous administration								
14	0.600	0.450	0.8	0.6	79	11	82	6
15	0.200	0.450	0.6	0.4	91	12	90	7
16	0.165	0.390	0.2	<0.2	88	8	109	5
17	0.160	0.230	0.3	<0.2	106	7	109	6
18	0.350	0.410	0.7	0.5	106	5	104	5
19	0.250	0.175	0.2	0.2	73	9	89	6
20	0.165	0.240	0.5	0.4	68	10	75	6
21	0.108	0.155	0.5	0.3	70	6	84	7
22	0.155	0.150	0.4	0.3	78	10	99	8
23	0.160	0.270	0.6	0.5	98	11	84	6
Median	0.182	0.255	0.5	0.4	84	10	90	6

The median values were calculated for the samples having detectable levels of proteins. It should be noted that the different proteins have different detection limits: β -LG, 0.001 μ g/ml; Ova, 0.2 μ g/ml; BSA, 1 μ g/ml; and BIgG, 4 μ g/ml.

whereas BSA and IgG were retained at higher concentrations in the serum, median values 84 $\mu\text{g/ml}$ (68–106 $\mu\text{g/ml}$) and 90 $\mu\text{g/ml}$ (75–109 $\mu\text{g/ml}$), respectively. These results indicate that OvA and β -LG were eliminated more rapidly from the circulation. In the milk samples, the median level of BSA was 10 $\mu\text{g/ml}$ (5–12 $\mu\text{g/ml}$) and IgG was 6 $\mu\text{g/ml}$ (5–8 $\mu\text{g/ml}$). These levels were again higher than those of OvA and β -LG, which were 0.4 $\mu\text{g/ml}$ (0.2–0.6 $\mu\text{g/ml}$) and 0.255 $\mu\text{g/ml}$ (0.108–0.600 $\mu\text{g/ml}$), respectively.

DISCUSSION

From the results of this investigation, it is evident that proteins fed orally to lactating rats are transmitted still in immunoreactive form to the blood and milk. However, the individual differences were pronounced. In some serum and milk samples, OvA, BSA, and IgG could not be detected at all, probably due to methodological limitations of the immunoprecipitation methods used. In fact, when the more sensitive RIA method was used, β -LG could be detected in all samples, but in highly varying concentrations.

Oral feeding of the proteins appeared to give higher relative concentrations in the milk than did intravenous administration. The most pronounced difference between serum and milk levels was seen for OvA and β -LG in the orally fed group, where the concentration of these proteins in milk were higher than in serum. In addition, the absolute concentration of each protein in the milk of the orally fed group was considerably higher than that observed in the milk of the intravenously administered group. This difference was even more pronounced with respect to the large total amount of OvA and β -LG that was administered intravenously. Oral administration of BSA also resulted in a higher relative concentration in the milk (when detected) in the majority of the rats than did intravenous administration.

With respect to a milk/serum ratio estimated for the intravenously administered group, the smaller molecules appeared to pass more rapidly over to the milk than the larger molecules. These results agree with the proposal that the mammary gland acts as a molecular sieve for proteins in the serum (34). However, when intravenously administered, the larger molecules IgG and BSA were detected in higher absolute concentrations in the milk than were OvA and β -LG. This could be due to the low clearance rates of IgG and BSA from the serum in comparison to those of OvA and β -LG, judging from the serum values shown in Table 1. It is obvious, however, that

despite the higher clearance rate of OvA and β -LG from serum as seen in the intravenously administered group, these proteins can be found in relatively high concentrations in the milk when they are fed orally.

The observation that orally fed proteins appear to accumulate in the milk raises interesting questions about the underlying physiological mechanisms. Gastrointestinal processing of the protein may result in qualitative changes in the structure of and/or the charge on the protein. Such changes could enhance the transmission of proteins from serum to milk. In support of this, crossed-immunoelectrophoresis revealed that BSA in the milk and serum samples contained a small fraction with a somewhat reduced electrophoretic mobility in comparison with that of the fed protein. However, this pattern was identical for serum and milk. Another factor that could be of importance for the availability of proteins to the mammary gland is the distribution of the absorbed proteins between the lymph and the blood circulations. The extravascular circulation is probably the primary location for the absorbed macromolecules from the intestine (35). Furthermore, it should be noted that once the proteins are deposited in the milk, they remain there and are probably not eliminated, whereas the proteins present in the serum are continuously in contact with the various elimination systems of the organism.

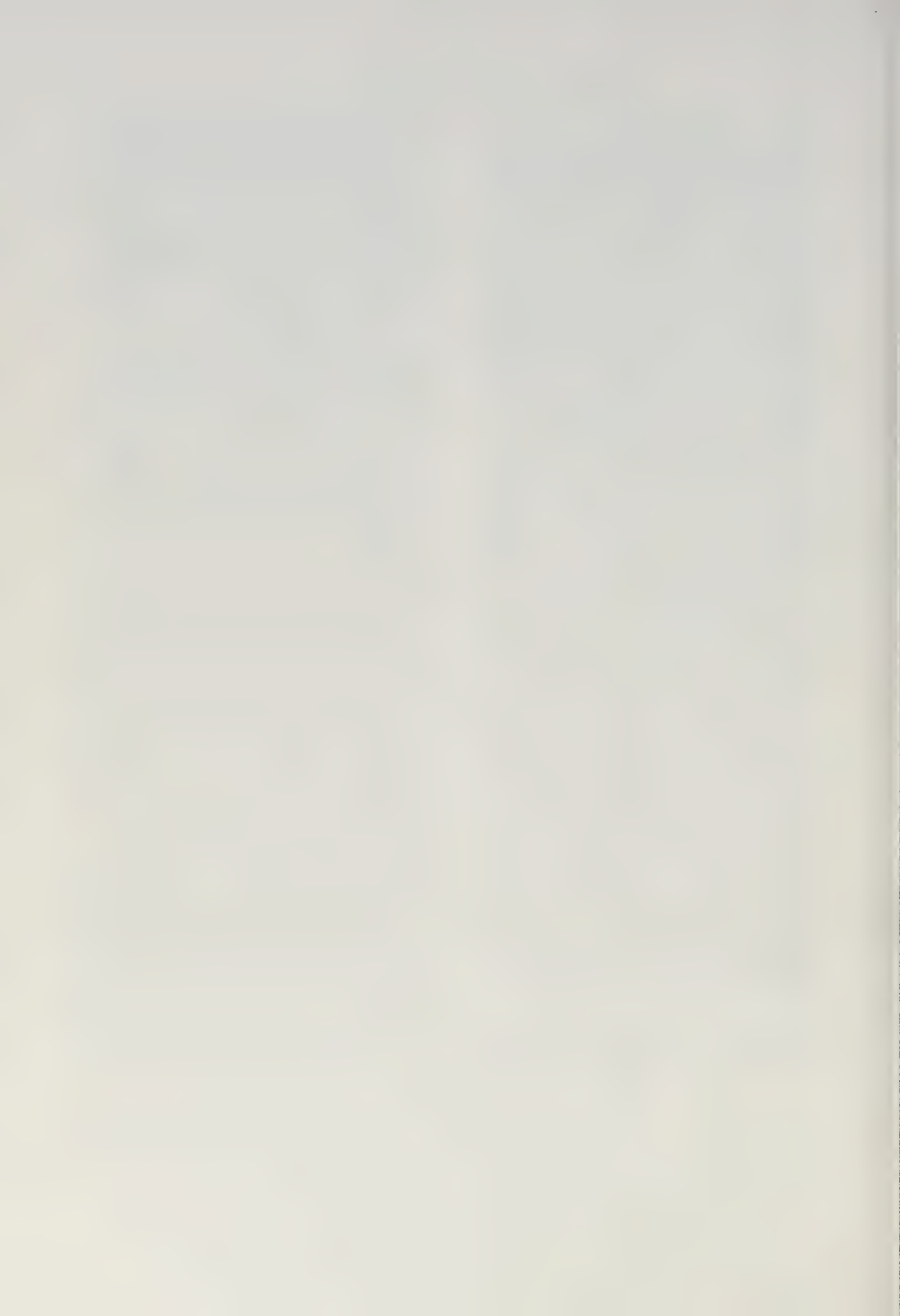
From the physiological point of view, it is tempting to speculate that this phenomenon is a naturally occurring event for the common proteins in the normal food of the rat. It has been shown that the i.p. injection of human gammaglobulins to lactating mice results in systemic tolerance in the nursing offspring specific to these proteins (36,37). It has also been shown that the specific protein content in the diet of the pregnant and lactating rabbit is of importance for the immunological response of the offspring, i.e., the offspring becomes immunologically tolerant to proteins included in their mother's diet (38,39).

In conclusion, these results suggest that the offspring of the rat and other mammals, via the milk, come in early contact with common potential antigens in the food of their mothers. This form of antigen presentation may produce immunological tolerance in the gut to "harmless" antigens in the normal food of the rat. We have recently shown that this presentation of common food antigens may begin even before birth (1).

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V

**Maternal Dietary Antigens and the Immune
Response of the Offspring in the Guinea Pig.**

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Summary

Guinea pig dams and their litters, were raised on either cow's milk protein-containing diet (MCD) or a milk free diet (MFD). At 8 w of age all litters were challenged i.p. with 50 μ g milk whey protein concentrate and 100 mg $\text{Al}(\text{OH})_3$ in saline. The immune response was estimated two weeks later as the titres of serum IgG antibodies against V67, β -lactoglobulin (β -LG) and α -lactalbumin (α -LA) using enzyme-linked immunosorbent assay (ELISA) and the tracheal Schuize-Dale response to these antigens.

Feeding milk protein antigen to dams from birth and during pregnancy induces antigen specific hypo-responsiveness (tolerance) in their offspring, despite no direct contact between the offspring and the milk proteins. Tolerance seems to be induced by the antigen itself since withdrawal of the MCD 10 d before delivery reduced tolerance in the offspring. No tolerance was produced in the offspring of dams fed the antigen from adulthood. β -LG appears to be the major antigen in milk whey since there was almost no antibody or tracheal response to α -LA in any of the animals tested.

The results indicate that maternal antigen experience and antigens present during pregnancy are important for the subsequent response to these antigens in her offspring.

Introduction

It is essential for the immune system to be able to discriminate between "harmless" and potentially "harmful" antigens. The gastrointestinal tract is exposed to numerous antigens to which an actively enhanced or suppressed immune response is maintained.

Antigens presented orally during the neonatal period and to adults have been shown to induce a state of antigen-specific immunologic hypo-responsiveness (tolerance) in many different species (Filipp 1965, Thomas & Parrot 1974, Hanson et al 1977, Kagnoff 1978, Coombs et al 1978, Swarbrick et al 1979, Strobel et al 1982, Heppel & Kilshaw 1982, Stokes et al 1983). Tolerance is a result of an active suppression maintained by antigen-specific T-suppressor cells (Richman 1978, Ngan & Kind 1978, Waters 1979, Mowat et al 1982, Ferguson et al 1983) and includes both cellular and humoral immune responses.

Guinea pigs are easily tolerized when fed an antigen early in life, but tolerance is more difficult to produce with growing age (Coombs et al 1978, Heppel & Kilshaw 1982) while in adult guinea pigs sensitization by the oral route is a well-known phenomenon (Devey et al 1976, Coombs et al 1978).

There have been few reports on maternal antigen experience and the subsequent response in the offspring. Halsey & Benjamin (1976) showed that tolerance was induced in mice born to a mother injected with human gamma-globulins. Pathriana et al (1981) showed tolerance induction to soya protein in the offspring of rabbits fed soya and Peri & Rothberg (1981) to BSA.

In the present study, the immune response to cow's milk proteins in the offspring of guinea pig dams fed milk-containing diet from early in life, was studied.

Materials and methods

Animals and experimental protocol

Eight female Guinea pigs of the Dunkin Hartley strain were delivered by caesarian section at 63 days of fetal age (2-3 days

before normal delivery). They were kept with a lactating foster mother and were raised to become the dams. Four of these were fed 4ml/100g b.w./day for 2 days of a cows milk whey protein concentrate (V 67, Semper, Sweden) solution, 50 mg/ml in saline by stomach tube. From their first day of life they had ad lib access to a pelleted experimental milk containing diet (MCD) made from normal guinea pig diet (K1, Ewos, Södertälje, Sweden) but with the fish protein replaced with V 67 which made up 70 % of the total protein content. Another four females were fed saline by stomach tube as above and had ad lib access to normal milk-free diet (MFD). One additional female (fed MFD from birth) was shifted from MFD to MCD at three months of age. At 6 months of age these 9 females were mated and their offspring were divided into 6 different groups based on their own and their dams diet (A-E plus controls) according to the protocol shown below. All groups had access to their diet until the end of the experiment.

Experimental group	n	Litters fed	Dams fed
A	5	MFD	MCD (until delivery, then MFD)
B	2	MCD	MCD
C	6	MCD	MFD
D	4	MFD	MCD (until 10 d before delivery, then MFD)
E	4	MFD	MFD (until 3 months of age, then MCD until delivery)
controls	10	MFD	MFD

At 3 months of age all animals in the second generation (group A-E and controls) were challenged by an i.p. injection of 50 μg V67 + 100 mg $\text{Al}(\text{OH})_3$ in 0.5 ml of saline prepared 1 hour before injection. Two weeks after injection the animals were killed using CO_2 , blood was taken by cardiac puncture and the trachea was dissected out. The blood was centrifuged at 3000xg and the serum was saved at -20°C until analysis.

Assay

Specific IgG, IgM and IgA titres to V67, β -lactoglobulin (β -LG) (Sigma, No.L-2506, St. Louis, Mo., USA) and α -lactalbumin (α -LA) (Sigma, No.L-4379) were estimated using an enzyme-linked immunosorbent assay (ELISA)(Engvall 1980). Chicken ovalbumin (OVA)(Sigma, No. A 5503) was used as a negative control. Microtitre plates (Immunoplatte 1, Nunc, Roskilde, Denmark) were coated over night at room temperature (RT) with 200 μl antigen (1 $\mu\text{g}/\text{ml}$) in a carbonate buffer solution (pH 8.3). After washing with phosphate-buffered saline containing 0.05% Tween 20 (PBS+Tween) for 3x2 min, all wells were filled with 200 μl PBS+Tween and 50 μl of sample was placed into each well of the top row. The samples were then serially diluted vertically down the plate (1/5 steps for IgG, 1/3 steps for IgM and IgA) to a final dilution of 1/390625 or 1/10935. The plates were then incubated in a humid chamber at $+4^\circ\text{C}$ for 16 h. After washing (as above) 200 μl of either rabbit anti-IgG (dil. 1/20000), IgM (dil.1/2000) or IgA (dil. 1/2000)(Miles, Nos.65-119, 64-322-1, 64-323-1, Slough, UK) was added. All dilutions were made in PBS+Tween. The plates were left in humid chambers at RT for 90 min. After washing 200 μl of peroxidase-conjugated swine anti-rabbit IgG (Dako No P217, Copenhagen, Denmark) diluted 1/1000 in PBS+Tween and containing 1 % normal pig serum was added. After incubation for two hours at RT

the plates were washed and 200 μ l of substrate solution (2,2'-azino-bis(3-ethylbenzthasoline sulfonic acid (ABTS), 1mg/ml, Sigma No. A1888) was added. After 60 min at RT the plates were read in an ELISA scanner (Titertek multiscan, Flowlab, Herts, UK) at 405 nm and the optical densities (OD) were recorded. The titres were expressed as an endpoint titre where the OD value differed 0.1 units from the background value of a normal guinea pig serum.

The tracheal response to the antigens was recorded by an in vitro challenge using a slightly modified Schultz-Dale (Schultz 1910, Dale 1913) set up and the response was related to that of histamine (Sigma, No. H7250), 10^{-3} mg/ml, according to the following formula:

$$\frac{\frac{1}{\text{antigen deflection in mm}}}{\frac{1}{\text{histamine deflection in mm}}} \times \text{antigen conc. mg/ml} \times 10^{-3} \text{ mg/ml}$$

Results

As seen in fig 1 and 2 guinea pigs born to mothers fed a cow's milk protein based diet (MCD) (group A) showed a significantly reduced IgG titre and tracheal responsiveness to the whole skimmed milk powder V67, as well as the individual protein β -LG ($p < 0.05$) compared to controls born to mothers on a milk-free diet (MFD). Feeding both the mothers and the offspring the MCD (group B) or by feeding just the offspring MCD from birth (group C) gave the same result.

Changing the mothers diet from MCD to MFD 10 days before deli-

very caused an intermediate antibody response in the offspring (group D) and the titre to V67 and β -LG were significantly ($p < 0.05$) lower than the controls (Fig 1.). All animals in this group showed tracheal response (Fig. 2).

Introducing the MCD to adult (3 month old) mothers did not significantly alter the IgG response in the offspring (group E) compared with the controls and all animals showed a tracheal response (Fig. 2).

The response to β -LG alone was almost identical to that of the whole milk whey (V67) in all animals, while no response to α -LA was seen in the Schulz-Dale test in any of the animals and only very low titres of IgG antibodies were found in four of the controls (data not shown).

Specific IgM antibodies were only detected in a few of the control animals and in two of the animals from group E, but in low titres not exceeding 1/4000 (data not shown). Serum IgA antibodies to milk proteins were not detected in any of the animals from the different groups.

Discussion

The results of the present investigation clearly show that by including protein antigens in the mother's diet it was possible to alter the immune response to these antigens in the offspring. The offspring show a decreased serum IgG titre and tracheal response after challenge despite no earlier direct contact with the antigens. The results indicate that the tolerant state is produced by the antigen itself, since withdrawing the antigen ten days before delivery markedly reduces the tolerance seen in the

offspring (group D). The results in group C show that feeding the milk protein (MCD) to guinea pig from birth makes them tolerant to milk proteins, which implies that the dams fed MCD from birth also are tolerant to V67. This was, however, not tested. Since feeding the MCD to a dam from adulthood did not tolerize her offspring (group E) it seems to be crucial how the dam herself has been exposed to and handled the antigen. This observation was only made in the offspring of a single dam and should therefore be interpreted with caution.

There has been no attempt to discriminate between the IgG and the IgE responses, but since the IgG levels and the Schulz-Dale response show the same pattern we assume that the tolerance seen also includes the suppression of the IgE response.

The finding that α -LA is a poor antigen in guinea pigs is consistent with the findings of other investigators (Ratner et al 1958, Devey et al 1976) and could be due to similarities with the homologous α -LA found in guinea pig milk. β -LG, on the other hand, produced a vigorous response equal to that of the whole preparation (V67), indicating that β -LG is the major antigen in V67. β -LG is only found in ungulate milk and the lack of a homologous protein in guinea pig milk might explain its immunogenicity in Guinea pigs. In clinical studies of cows milk protein (CMP) intolerance β -LG has been shown to be a major allergen (Freier et al 1969, Kuitunen 1975).

Assuming that it is the presence of the antigen itself that produces the tolerance in the offspring it is interesting to speculate on what route the antigen takes. Other observations (Telemo in prep.) made in our laboratory show that by a single feed of CMP to pregnant Guinea pigs we were able to detect small amounts of immunologically reactive milk proteins in the amniotic

fluid. In the pregnant and lactating rat we have been able to detect CMP in the amniotic fluid (Dahl 1984) and in the milk (Tellems 1986) afterfeeding with CMP. We have also found that CMP deposited in the amniotic fluid are effectively absorbed by the fetuses and transmitted into their blood stream.

Oral tolerance is believed to be an active state and apparently there are several different mechanisms involved in its induction and maintenance. The nature of the antigen and the dose administered are important factors (Tomasi 1980). The biological age of the organism and hence the maturation of the immune system seems crucial for the ability to show tolerance (Hanson 1981, Strobel & Ferguson 1984) and it is necessary to consider the stage of maturation when comparing neonates from different species. The guinea pig is relatively mature at birth and starts almost immediately to eat solid food in addition to the mothers milk. If the immune system is to be prepared for this immediate massive antigen exposure the "schooling" must take place already in utero, which is supported by the present results.

Bruce and Ferguson (1986) found that serum from mice fed ovalbumin (OVA) produced suppressed DTH response when injected into a recipient mouse whilst injecting OVA directly failed to produce this response. The altered response to "biologically filtered" antigens might contribute to the effectiveness of the "transfer" of tolerance from mother to offspring seen in this study.

With the growing evidences of an active priming of the offspring via maternal antibodies (idiotypes, anti-idiotypes and immune-complexes) (Rubinstein et al 1982, Stein & Söderström 1984, Jarret & Hall 1982), it is important to consider the immune response produced in the dams. It is therefore likely that introducing the antigen to an adult guinea pig dam produces a different

response than feeding her from birth, as evaluated by the altered response in her offspring seen in this investigation (group E).

In conclusion we propose that maternal antigen experience is of importance for the immune response to antigens in her offspring. In general we can speculate that a tolerant mother gives birth to a tolerized offspring.

Aknowledgement

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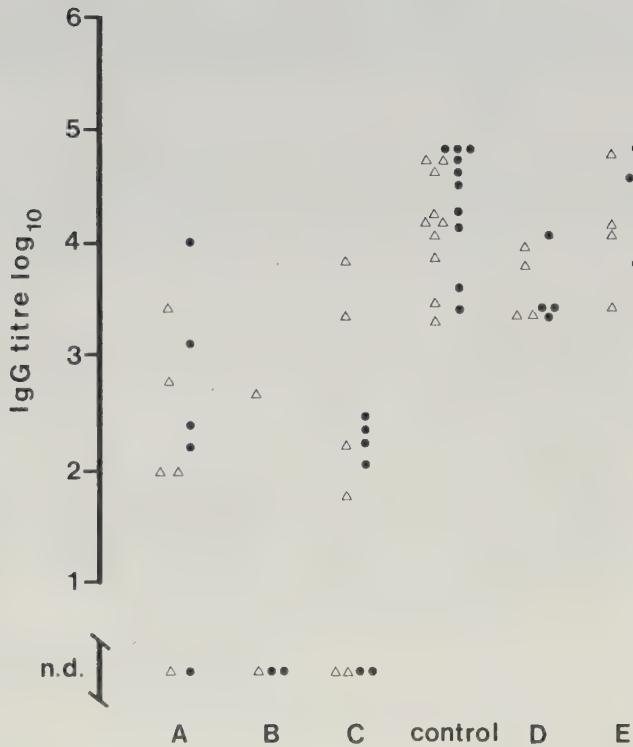


fig.1

The specific serum IgG titre ($\log_{10}$) against V67 and β -LG in guinea pigs two weeks after i.p. challenge with 50 μ g of V67 and $\text{Al}(\text{OH})_3$ 100 mg in saline. Group A=MFD fed born to dams fed MCD from birth; B=MCD fed born to dams fed MCD from birth; C=MCD fed born to dams fed MFD from birth; control=MFD fed born to dams on MFD from birth; D=MFD fed born to a dam fed MCD from birth until 10 d before delivery and then MFD; E=MFD fed born to a dam fed MCD from 3 months of age.

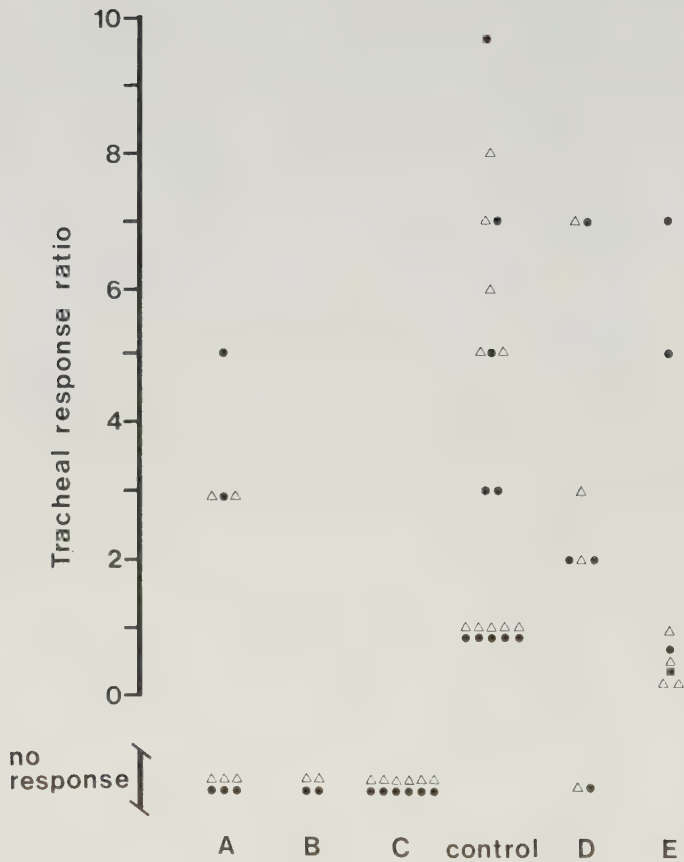


fig.2

The in vitro tracheal response to V67 and β -LG as related to the response to 10^{-3} mg/ml of histamine in guinea pigs, two weeks after i.p. challenge with 50 μ g of V67 and 100 mg $Al(OH)_3$ in saline. Group A=MFD fed born to dams fed MCD from birth; B=MCD fed born to dams fed MCD from birth; C=MCD fed born to dams fed MFD from birth; control=MFD fed born to dams on MFD from birth; D=MFD fed born to a dam fed MCD from birth until 10 d before delivery and then MFD; E=MFD fed born to a dam fed MCD from 3 months of age.

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